EVALUATION OF ANALGESIC AND ANTICONVULSANT ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *COSTUS PICTUS* LEAVES IN WISTAR ALBINO RATS

Dissertation

Submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

In partial fulfilment of the requirements for the award of the degree of

M.D. PHARMACOLOGY

Branch VI

APRIL 2016

EVALUATION OF ANALGESIC AND ANTICONVULSANT ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *COSTUS PICTUS* LEAVES IN WISTAR ALBINO RATS

Dissertation

Submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

In partial fulfilment of the requirements for the award of the degree of

M.D. PHARMACOLOGY

Branch VI

APRIL 2016

CERTIFICATE

This is to certify that this dissertation entitled "**Evaluation of Analgesic and Anticonvulsant Activity of Hydroalcoholic Extract of *Costus pictus* Leaves in Wistar Albino Rats**" is a bonafide record of the work done by **Dr. Anandhalakshmi. A** under my guidance and supervision in the Department of Pharmacology during the period of her postgraduate study for **M.D. Pharmacology [Branch – VI]** from 2013-2016.

Dr. Rema Menon N, M.D.,
[Guide]
Professor and Head,
Department of Pharmacology,
Sree Mookambika Institute of Medical Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu – 629 161.

Dr. Madhavrao, M.D.,
[Co-Guide]
Assistant Professor,
Department of Pharmacology,
Sree Mookambika Institute of Medical Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu – 629 161.

Mr. Sarath Babu Kurra, M. Sc.,
[Co-Guide]
Assistant Professor,
Department of Pharmacology,
Sree Mookambika Institute of Medical Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu – 629 161.

Dr. Rema. V. Nair, M.D., D.G.O.,
Director
Sree Mookambika Institute of Medical Sciences, Kulasekharam,
Kanyakumari District,
Tamil Nadu – 629 161.

DECLARATION

I, Dr. Anandhalakshmi. A hereby submit the dissertation entitled "**Evaluation of Analgesic and Anticonvulsant Activity of Hydroalcoholic Extract of *Costus pictus* Leaves in Wistar Albino Rats**" done in partial fulfilment of **M.D. Pharmacology [Branch-VI]** in Sree Mookambika Institute of Medical Sciences, Kulasekharam. This is an original work done by me under the guidance and supervision of Dr. Rema Menon N.

Dr. Rema Menon N, M.D.,
Professor and Head
Department of Pharmacology
Sree Mookambika Institute of
Medical Sciences
Kulasekharam [K.K District]

Dr. Anandhalakshmi. A
Post graduate
Department of Pharmacology
Sree Mookambika Institute of
Medical Sciences
Kulasekharam [K.K District]

EVALUATION OF ANALGESIC AND ANTICONVULSANT ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *COSTUS PICTUS* LEAVES IN WISTAR ALBINO RATS

Dissertation

Submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

In partial fulfilment of the requirements for the award of the degree of

M.D. PHARMACOLOGY

Branch VI

APRIL 2016

Acknowledgement

I express my heartfelt gratitude to **Dr. Rema Menon N, Professor and Head of Department of Pharmacology** and my guide for her valuable suggestions, critical views and constant motivation throughout the study period. She lent her full support in times of difficulties that I encountered during this study period and I thank her for all the help rendered to me in completing my study. I sincerely thank her for the valuable help, suggestions and supervision throughout the study. I express my gratitude for her constructive criticisms and pains she had taken for completion of this work.

I sincerely thank my Co-guide **Dr. Madhavrao** for his tremendous help and guidance throughout the study. His constant encouragement has been indispensible to the completion of the work. I once again thank him for his full support and patience he had shown in times of obstacles that I encountered during the study. I also thank him for helping me with the statistical analysis of the study.

I express my gratitude to **Mr. Sarath Babu Kurra** my Co-guide who gave his valuable suggestions, critical views and comments at each stage of my dissertation work. I acknowledge with gratitude the immense help and support he showed to me for completing my study.

I extend my thanks to my Professors **Dr. Reneega Gangadhar** and **Dr. V. P. Bhalla** and Associate Professor **Dr. Ganesh. V,** for their motivation and support. I

also thank my Assistant professors **Dr. Shruthi R** and **Dr. V. M. Sandeep** for their guidance and suggestions during the study.

I extend my sincere and heartfelt thanks to our Chairman **Dr. Velayuthan Nair** and Director **Dr. Rema V Nair** for giving me an opportunity to conduct the study. I humbly thank them for providing me the facilities to carry out my study and also for being a source of inspiration. I would also like to thank the Principal of the Institution **Dr. Padmakumar** for his support in accomplishment of my dissertation work.

I express my heartfelt thanks to **Mr. Muthusamy** for the timely authentication of my plant specimen.

I specially thank my colleague, **Dr. Prathab Asir. A,** for his immense help, comments and suggestions during each stage of my study. His valuable and timely help made me complete my study on time. His constant encouragement was an immense source of motivation during the hardships I faced during the study.

I am grateful to the other postgraduates in my department **Dr. Biacin Babu, Dr. Parvathy RL, Dr. Shanthi M, Dr. Navaneeth A, Dr. Arjun G Nair, Dr. Sushmita Ann S. J and Dr. Suhaina. A,** for their help in various technical aspects of my study.

I also thank my department office staff **Mrs. Florence Vimala and Miss. Sangeetha** for the support they have extended to me. I am also thankful for the staff at the animal house for all their timely help.

TABLE OF CONTENTS

LIST OF TABLES

Table of contents

LIST OF FIGURES

LIST OF IMAGES

Evaluation of Analgesic and Anticonvulsant Activity of Hydroalcoholic Extract of *Costus Pictus* Leaves in Wistar Albino Rats

Abstract

Background:

Oxidative stress has a significant role in the development, progression and treatment refractoriness of various pathophysiological conditions including pain and epilepsy. *Costus pictus* D. Don commonly referred as insulin plant has proven antioxidant property. Phytochemical studies of this plant have confirmed the presence of potent antioxidants mainly flavonoids. With huge evidence in literature for the role of oxidative stress in pain and epilepsy, this study was planned to evaluate the analgesic and anticonvulsant activities of hydroalcoholic extract of *Costus pictus* leaves in animal models which have not been studied so far.

Aims and objectives:

To evaluate the analgesic and anticonvulsant activities of hydroalcoholic extract of *Costus pictus* leaves in Wistar albino rats.

Materials and methods:

A total of 78 healthy Wistar albino rats (180-250 g) of either sex were included in the study and divided into 13 groups (Group I to XIII) consisting of 6 animals each. The hydroalcoholic extract of *Costus pictus* leaves [CP-HALE] was prepared using cold maceration method and was tested at two doses (200 mg/Kg BW and 400 mg/Kg BW) given orally.

Central analgesic activity was tested using tail clip and hot plate methods [standard - morphine 5 mg/Kg BW i.p.] and peripheral analgesic activity was evaluated using writhing test [standard – morphine (5 mg/Kg BW i.p.) and diclofenac sodium (12.5 mg/Kg BW orally)]. Anticonvulsant activity was evaluated using Maximal Electroshock induced Seizure (MES) model [standard - phenytoin 10 mg/Kg BW; i.p.] and pentylenetetrazole (PTZ) model [standard - sodium valproate 400 mg/Kg BW i.p.].

Results:

With CP-HALE 400 mg/Kg BW, there was a significant increase in reaction time in tail clip and hot plate methods. There was also significant reduction in total number of writhes and the percent inhibition of writhes [82.87%] was comparable to the standard drugs.

MES model revealed that at both the doses (200 mg/Kg BW and 400 mg/Kg BW) of CP-HALE there was 100 percent protection against tonic hind limb extension (THLE). A significant decline in the seizure scores was also seen at 400 mg/Kg BW.

In PTZ model, both the doses of CP-HALE increased the time for onset of seizures and decreased the duration of seizures which were higher and significant at 400 mg/kg BW and at this dose there was also a significant reduction in the total number of seizures (in one hour). There was no significant decline in the seizure scores at both the test doses (200 mg/Kg BW and 400 mg/Kg BW).

Conclusion:

The hydroalcoholic extract of *Costus pictus* leaves possess significant analgesic and anticonvulsant activity in Wistar albino rats at the dose of 400 mg/Kg BW.

Key words: Pain, Seizures, Epilepsy, Oxidative stress, Antioxidant, Insulin plant, hydroalcoholic extract

1. Introduction

The practice of using natural products in therapy is well established since ancient times. Products from natural sources also serve as lead compounds for design and synthesis of new drugs. Plants contribute for around 25% of the drugs used world-wide and still remains the major resource for new drug development.[1]

Costus pictus D. Don (syn. *Costus mexicanus* Leibm.), commonly called as insulin plant,[2] has its origin in Mexican moist forests and hills.[3] It has gained popularity in India recently. It is a perennial herb[3] which is grown as an ornamental plant in South Indian gardens, particularly in various parts of Kerala.[2] It belongs to the family *Costaceae*[2] and is very popular for its antidiabetic property for which its fresh leaves are chewed and eaten raw by diabetic people.[4] There are several previous studies which have demonstrated the various medicinal properties of *Costus pictus* such as antidiabetic,[4,5] antihyperlipidemic,[5] antibacterial,[6,7] anthelmintic,[2] analgesic,[3] antioxidant[5,8,9] and anticancer activity.[10] *Costus pictus* extract (500-2000 mg/day in humans, orally) has been patented for glycemic control in diabetes mellitus.[3] In Cuba, the decoction of its fresh leaves and stems has been a traditional therapy for various urinary disorders and also used as a pain relief in renal colic.[3]

Pain accompanies almost all pathological conditions and is an unpleasant sensation often accompanied by anxiety and an urge to terminate the feeling immediately.[11,12] It often requires rapid and effective treatment with

analgesics. Commonly used analgesics are opioids and non-opioids [Nonsteroidal Anti-Inflammatory Agents (NSAIDs) and antipyretic-analgesics].[12] Opioids remain the most potent and reliable centrally acting analgesics and are considered the mainstay in pain management.[11,12] Their use is limited by its side effects and addiction liability.[11] The NSAIDs which are usually used for mild to moderate pain is also limited by its adverse effects, including gastrointestinal, renal and cardiac effects.[13] There is still a growing need for an effective analgesic which is better tolerated. A study using mice models has proved a significant and dose-dependent peripheral and central analgesic activity of *Costus pictus* decoction.[3] Hence, it is rational to carry out further studies to demonstrate its analgesic studies using different animal models to validate the evidence.

Epilepsy is a clinical condition in which an individual presents with recurrent abnormal or excessive neuronal activity due to a chronic underlying pathology.[14,15] Epilepsy occurs in various forms and multiple causes underlie its pathogenesis. 5-30 persons per 1000, is the estimated prevalence for epilepsy.[14] The outcome of treatment is largely based on selection of an appropriate therapy. Several antiepileptic drugs that are currently available control seizure activity rather than treating the underlying disorder.[15] Their usefulness is limited due to their toxicity and development of drug resistance.[14,15,16] The search for more effective and less toxic antiepileptic drugs still remains valid. There is a strong correlation between oxidative stress and pathogenesis of epilepsy.[17,18] It is thus rational to

hypothesize that *Costus pictus* plant extract which has significant antioxidant activity[5,8,9] might be beneficial in the treatment of epilepsy. Also, there are no studies so far to evaluate the analgesic and anticonvulsant activity of hydroalcoholic extract of *Costus pictus* leaves.

There is a growing interest on the therapeutic potential of natural products in recent years and pharmacological screening of plants is considered as a suitable approach to new drug development.[1] In the present study, hydroalcoholic extract of *Costus pictus* leaves were chosen to explore its analgesic and anticonvulsant properties which have not been screened so far.

HYPOTHESIS

&

JUSTIFICATION

2.1 Hypothesis

Hydroalcoholic extract of *Costus pictus* leaves possess analgesic and anticonvulsant properties.

2.2 Justification

Oxidative stress generates free radicals[18] and has been implicated in the development, progression and treatment refractoriness of various pathophysiological conditions including pain[19,20] and epilepsy.[17,18,21] Antioxidants are endogenous or exogenous substances which act by reducing free radical formation, free radical scavenging or removal of free oxygen.[22] Oxidative stress and mitochondrial dysfunction is implicated in neuronal death, hence has important role in the onset and maintenance of seizures.[17,18] It was shown that partial prevention of induced seizures is possible with the use of antioxidants.[18] Currently available analgesics and antiepileptic drugs, have limitations such as toxicity, teratogenicity, drug resistance, and significant pharmacokinetic interactions.[15] Hence, antioxidants may be an important adjunct to conventional therapies[17] and also they serve as a direction for development of novel antiepileptic drugs.[18]

The antioxidant property of *Costus pictus* leaves[5,8,9] and also its various other parts[2,6] has been proved by several previous studies. Various studies on the phytochemistry of *Costus pictus* have revealed that it contains compounds such as coumarins, flavanoids, phenols and

lactones which are responsible for its antioxidant[6,8] and analgesic properties.[3] It was also shown in an earlier study that the dry hydroalcoholic extract of *Costus pictus* leaves possessed highest antioxidant activity.[9] A dose dependent central and peripheral analgesic effect of *Costus pictus* decoction has been proved in mice models.[3] There is a need for demonstrating analgesic activity in different animal models for validating the evidence.

There are no studies so far to evaluate the analgesic and anticonvulsant activities of hydroalcoholic extract of *Costus pictus* leaves in animal models. With a large body of evidence for the role of oxidative stress in pain and epilepsy, it is thus rational to evaluate the analgesic and anticonvulsant effects of *Costus pictus* which has proven antioxidant property.

AIMS

&

OBJECTIVES

3. Aims and Objectives:

To evaluate the following activities of hydroalcoholic extract of *Costus pictus* leaves in Wistar albino rats

i. Central analgesic activity using tail clip and hot plate methods

ii. Peripheral analgesic activity using 4% NaCl induced writhing test

iii. Anticonvulsant activity on electrically induced seizures

iv. Anticonvulsant activity on pentylenetetrazole induced seizures

REVIEW OF LITERATURE

4. Review of Literature

4.1 Pharmacognosy of *Costus pictus*

Pharmacognosy is a multidisciplinary science which plays a significiant role in the drug discovery and development. The term *pharmacognosy* is derived from the Greek words "*pharmakon*" meaning drug and "*gnosis*" meaning knowledge. This term was first introduced by an Austrian physician Schmidt in 1811.[23] The American Society of Pharmacognosy defines pharmacognosy as "the study of the physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources." Ethnobotany, ethnomedicine, and ethnopharmacology are considered to be an integrative part of pharmacognosy.[23]

Costaceae which consists of four genera, is one of the most recognized families of the order *Zingiberales*.[24] It is differentiated from other families by their well-developed aerial shoots with a spiral phyllotaxy.[24] *Costus* being the largest genus of this family, consists of more than hundred different species which are found in various tropical countries around the world.[24,25] Around 5 species are seen in southeastern Asia.[24]

Costus pictus D. Don (synonym - *Costus mexicanus* Liebm.) is one of the medicinally important plants with its origin in South and Central

America.[26] It is widely grown as an ornamental plant in Indian gardens, especially in Kerala.[27] *Costus pictus* D. Don belongs to the family *Costaceae* and is differentiated from the *Zingiberaceae* family by the spirally arranged leaves and the absence of aromatic essential oils in its rhizomes.[28] Because of the spiral arrangement of its leaves it is commonly referred as spiral ginger or step ladder.[28] This plant has gained wide popularity in India as a low cost herbal remedy for diabetes.[29] Fresh raw leaves of *Costus pictus* are chewed daily[30] and are considered as a munching dietary supplement for diabetes.[31] Though commonly used, no formulation of this plant is currently marketed.[31]

4.1.1 Taxonomical hierarchy[32]

Kingdom	:	Plantae
Phylum	:	Magnoliophyta
Class	:	Liliopsida
Order	:	Zingiberales
Family	:	Costaceae
Genus	:	*Costus*
Species	:	*Costus pictus* D. Don

A recent phylogenetic analysis of the *Costaceae* family showed *Costus* is polyphyletic and the taxonomy has been revised. Three new genera have been seggregated from *Costus: Paracostus, Cheilocostus* and

Chamaecostus. *Costus* is now considered as a much smaller genus with restricted diversity.[24]

4.1.2 Habitat and distribution

Costus pictus is a perennial herb which is native to Mexico.[3] It is widely distributed along the coastal regions from Mexico to Costa Rica.[5] *Costus pictus* or spiral ginger[7] is called by different local names in different regions. It grows in moist forests, hill forests, clearings, along waterways and roads at sea level of 300-1800 meters.[3] It is also grown in various tropical countries and is recently introduced in India.[5] It is grown widely in south Indian gardens and also runs wild in many places.[33]

4.1.3 Growth and propagation[34]

Spiral ginger or spiral flag grows well either in full sun or partial shade. It requires fertile soil with adequate moisture and hence often planted near water sources. Propagation is usually done by the division of clumps, cuttings, or by separating the offsets and plantlets that form below the flower heads.

4.1.4 Morphological features of *Costus pictus* [3,5,25,35-37]

Costus pictus is a perennial rhizomatous herb which has erect or spreading stems and grows to a height of 2-3 meters and spreads to around 1.5-2 meters. The leaves are simple, glossy, linear with wavy margins and are spirally arranged around the stem. The narrow elliptic or obovate shaped leaves are subsessile and dark green in colour. Globose

and ovoid inflorescences appear as terminal spikes around 3-8 cm in length. Ovate bracts forms a cone like structure which is green colored in exposed part and reddish in covered part. The corollas are yellow with red stripes while the labellum is yellow. Anther is cream colored. Flowers do not produce any aroma. Fruit is an ellipsoidal capsule.

4.1.4.1 Macroscopic features of *Costus pictus* leaf[35-37]

The leaves of *Costus pictus* are elliptical 7.9 – 15.5 cm long and 3.8 – 5.7 cm broad. They are firm, flexible, and slightly succulent with entire margins and parallel venation. Apex is acute to acuminate and the base is subsessile to cuneate. Leaves are leathery to coriaceous in texture. They are dark green adaxially and light green abaxially. The leaves have no odour but are slightly sour to taste. They are arranged in a spiral (monostichous) fashion.

4.1.4.2 Microscopic features *Costus pictus* leaf[5]

The leaves are amphistomatic with paracytic stoma. The following features can be seen in a transverse section of *Costus pictus* leaf:

a. Epidermis consisting of barrel shaped cells containing starch grains. Upper epidermis consists of simple unicellular, pointed non-glandular trichomes.

b. 2-3 layers of hypodermal parenchyma - below the upper epidermis and above the lower epidermis. Many crystalline inclusions were seen in most hypodermal cells.

c. Mesophyll containing spongy tissues (between hypodermal layers). The mesophyll consisted of closely packed cells containing starch grains.

d. No palisade layer

e. A single layer of sclerenchyma was seen enclosing the vascular bundle.

4.1.5 Phytochemistry

Around 18 chemical constituents have been identified and analyzed from *Costus pictus* leaves.[28]

George et al.[38] performed phytochemical investigation of air dried leaves of *Costus pictus*. A preliminary physico-chemical analysis revealed high fibre content of the leaves (21.1%), which is essential in its activity against diabetes. A qualitative ash analysis of air dried leaves showed the presence of anions (carbonate, oxalate, chloride, phosphate and sulphate) and cations (sodium, potassium, magnesium and iron). Successive extractions of leaves with different solvents (petroleum ether, cyclohexane, acetone, ethanol) and its analysis showed presence of steroids in all the studied extracts. α-tocopherol present in ether fraction may be responsible for the antioxidant property of the leaf extract.

Shiny et al.[39] performed phytochemical analysis of 24 different extracts yielded by two different extraction procedures – cold maceration and hot continuous extraction (soxhlet method). Hexane, ethyl acetate, methanol and aqueous extracts of leaves, stem and rhizomes were taken

for the analysis. The maximum extract yield was obtained from the leaves in both extraction procedures. Steroids, triterpinoids, alkaloids, phenols, glycosides, quinones, coumarins and flavanoids were found by preliminary phytochemical tests of extracts from different parts. Yield and concentration of phytochemicals and secondary metabolites was higher in the methanolic extract of leaf obtained from cold maceration method when compared to soxhlet method. But the phytochemcial constituents were similar when obtained by these extraction methods which proved the absence of thermolabile consitutents in *Costus pictus.* The study also identified the presence of a glycoside (β- L- Arabinopyranose methyl glycoside) as the component responsible for the antidiabetic activity of the methanolic leaf extract. Another study by Shiny et al.[29] revealed that the phytochemical content and their potent hypoglycemic activity of the *Costus pictus* plant was not altered by the environmental factors.

Remya et al.[5] studied the *Costus pictus* leaves for the presence of flavanoids, phenolic acids, quinones and steroids. Analysis of flavanoids and phenoilc acids were performed using spectrophotometry and chromatography techniques. The flavanoids identified were Kaempferol, 4'- OMe- Kaempferol and 3', 4'- di OMe-quercetin. The phenolic acids identified on chromatographic analysis were 2, 5-dihydroxy benzoic acid, 3,5-dihydroxy benzoic acid, p-hydroxy benzoic acid, o-coumaric, cis and trans-p-coumaric acid, vanillic, syringic, melilotic, α-resorcyclic and gentisic acids. The leaves also showed the presence of saponins, tannins,

quinones and steroids while glycoflavones and alkaloids were not detected in the analysis of leaves.

Thomas et al.[2] performed the phytochemical analysis of hydro-alcoholic extract of rhizomes of *Costus pictus* D. Don. The results confirmed that flavanoids, phenolic compounds, tannins, saponin glycosides, carbohydrates and steroids were present in detectable amounts in the studied extract.

Sulakshana et al.[36] studied to quantify the amount of diosgenin present in the rhizomes and leaves of different species of *Costus* (*Costus pictus, Costus speciosus and Costus igneus*) using HPLC method. *Costus* species are known to contain diosgenin (a steroidal saponin) as a major bioactive component. This study revealed higher percentage of diosgenin in *Costus pictus* when compared to the other two species. Rhizomes of *Costus pictus* (2.54%) had higher diosgenin content than its leaves (0.83%).

A study by Jose et al.[26] analyzed the essential oils of the stems, leaves and rhizomes of *Costus pictus*. The constituents of essential oils of leaves were dodecanoic acid (3.96%), farnesyl acetone (7.04%), α-ionone (8.01%), β-ionone (8.69%), 2-pentanol (22.48%) and hexadecanoic acid (24.51%). Hexadecanoic acid (palmitic acid), the major constituent of *Costus pictus* leaf oil, by enhancing the LDL to HDL ratio in healthy adults, can result in the development of coronary heart diseases. The study concluded that the long term use of *Costus pictus* leaves for the diabetic

treatment can result in serious cardiac diseases and hence could not be used as a standard anti diabetic treatment.

4.1.6 Medicinal properties

The decoction of *Costus pictus* stems and leaves are used in Cuba as a traditional medicine in the treatment of urinary infections, renal colic and renal lithiasis.[3] Aerial parts of *Costus pictus* plant have been used as an infusion in Mexican folk medicine to treat kidney diseases[5, 34] and was also known to possess diuretic activity.[5] In India, especially Kerala, it is used as a herbal remedy for diabetes.[2] The plant has been found to possess various other pharmacological activities like hypolipidemic, diuretic, antioxidant, antimicrobial and anticancer activities. Its aqueous extract has shown inhibitory effect on the formation of calcium oxalate monohydrate and hence prevents urolithiasis. It has also been proved that its ethanolic extract has ameliorative effect on mitochondrial enzymes in alcohol induced oxidative stress.[34]

4.1.7 Toxicity studies

Remya et al[5] evaluated the acute toxicity of fresh aqueous leaf extract of *Costus pictus* on normal rats weighing 150-200 g in graded doses up to a maximum of 1g/Kg BW/day orally for 30 days. The study revealed that there was no effect on the appearance or general behavior of the animals. There were no untoward changes in food and water intake, body weight, body temperature and state of stool. No mortality was observed during the test period.

4.1.8 Pharmacological screening done with *Costus pictus*

4.1.8.1 Analgesic and anti-inflammatory activity

Moron et al[3] evaluated the analgesic and anti-inflammatory activities of *Costus pictus* decoction (30%) of fresh leaves and stems. A phytochemical screening of *Costus pictus* decoction and identification of the secondary metabolites were also performed in this study.

Peripheral analgesic response was demonstrated using 0.75% acetic acid induced writhing in mice. Control animals received distilled water. *Costus pictus* decoction (30%) of fresh leaves and stems were used in doses of 0.5, 1.0 and 5.0 g fresh plant material / Kg body weight (BW); orally fed via gastric feeding tubes. The decoction decreased the number of writhes significantly and in a dose-dependent manner over an observation period of 15 minutes. The central analgesic response was demonstrated in mice by tail immersion method. The time to response (tail withdrawal) was recorded. Only the highest dose of plant decoction (5.0 g/Kg BW) significantly increased the time to tail withdrawal. The results of the above analgesic models used in the study proved a significant and dose dependent inhibition of central and peripheral pain responses by *Costus pictus* decoction.

In the same study by Moron et al.[3] the anti-inflammatory property of as *Costus pictus* decoction was evaluated using cotton granuloma model in male Sprague-Dawley rats. Cotton pellets (50 g) were implanted on the dorsal side of the body and the wound was sutured.

Control group animals received sterile water. Test groups received aqueous extracts at doses of 0.5, 1.0 and 5.0 g fresh plant material / Kg BW in a single daily dose for four days. On 5th day, animals were sacrificed and excision of granulomas was done. The granulomas were placed in a ventilated oven at a temperature of 150±10 °C for three hours. Water content of the granuloma (wet weight minus dry weight) and fibrogranulosa content (dry weight of cotton pellet) in grams were assessed. The *Costus pictus* decoction did not modify significantly the water content; however the fibroganulosa content showed significant changes. This study thus demonstrated the lack of effect of *Costus pictus* decoction on inflammation induced by cotton granuloma model.[3]

The phytochemical analysis detected the presence of phenolic compounds, flavanoids, coumarins, saponins, lactones and reducing sugars in the aqueous extract. The presence of significant amounts of these compounds were attributed to be responsible for its analgesic property.[3]

4.1.8.2 Antioxidant activity

Rege et al[9] evaluated the *in vitro* antioxidant activity of fresh-aqueous, fresh-hydroalcoholic, dry-aqueous and dry-hydroalcoholic extracts from fresh and dry leaves of *Costus pictus* using 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. Freshly chopped or shade dried leaves were subjected for extraction. Hot decoction method was used to prepare aqueous extract while cold maceration using 20 % ethanol was

used for obtaining hydro alcoholic extract. Ascorbic acid at concentrations of 3-7 μg/mL was used as the standard. Among the four extracts studied, dry hydroalcoholic extract showed the strongest antioxidant activity. This study also assessed pepsin enzyme inhibitory activity of the extracts. All the extracts studied showed potent pepsin inhibitory activity, of which fresh-hydroalcoholic extract showed highest inhibition on pepsin enzyme activity.

Rajasekaran et al[8] studied the role of *Costus pictus* ethanol (CPE) extract against the oxidative stress caused by hyperglycemia in liver and kidney of alloxon administered rats. Glibenclamide (5 mg/Kg BW) was used as the standard drug for comparison. The CPE extract was administered orally in doses of 200 mg/Kg BW and 400 mg/Kg BW. The study revealed that the rats treated with CPE extract recovered from the altered antioxidant status due to hyperglycemia. GSH (reduced glutathione) levels were not significantly altered in both kidney and liver of CPE treated rats which indicate its protective role in oxidative stress. Also, protective effects of CPE extract on antioxidant enzymes [glutathione peroxidase (GPx), glutathione-s-transferase (GST), catalase (CAT), superoxide dismutase (SOD)] was noted in the kidney and liver tissues. High MDA level, an aldehyde product of lipid peroxidation in kidney and liver, is an indicator of oxidative stress induced by hyperglycemia. MDA being more stable and cytotoxic than reactive oxygen species (ROS) is an important marker enzyme for

oxidative stress severity. MDA levels were less affected in CPE administered rats. Also, there were no significant alterations in the marker enzymes (AST, ALT, ALP and LDH) in CPE treated rats. These were attributed to the protective role of the phytoconstituents of *Costus pictus* leaves against oxidative damage caused by hyperglycemia. With CPE treatment, glucose levels also returned to normal levels at the end of treatment period. This study confirmed the antioxidant and hypoglycemic potential of *Costus pictus*.

Remya et al[5] evaluated the antidiabetic activity of *Costus pictus* fresh leaf extract given orally to alloxan induced diabetic rats at doses of 100, 200, 400, 600 and 1000 mg/Kg BW for 60 days. The study concluded that there was a significant decrease in blood glucose levels, lipid profiles, urea, uric acid and creatinine at 200 and 400 mg/Kg BW. The effect at 400 mg/Kg BW was found to be equally effective as the standard drug Glibenclamide (600 μg/Kg BW). Treatment with fresh leaf extract significantly reduced TBARS levels in plasma, liver and kidney. There was also a significant increase in reduced glutathione (GSH) and vitamin C levels. The levels of SOD, CAT were raised while the levels of GPx were elevated in liver but reduced in kidney. The restoration of activity of these antioxidant enzymes after treatment with fresh leaf extract revealed its antioxidant potential. There were also changes in the levels of carbohydrate metabolizing enzymes. After treatment with extract, hexokinase activity was increased while activity

of glucose-6-phosphatase and fructose-1, 6-bisphosphatase was decreased which was comparable with the standard drug. The study claimed that these changes were due to increased insulin secretion stimulated by the treatment. The extract showed higher potency at a dose of 400 mg/Kg body weight. This study thus demonstrated that treatment with *Costus pictus* leaf extract resulted in prevention of glycation and improved the activity of enzymatic and non-enzymatic anti oxidants (decreased lipid peroxidation) along with a reduction in blood glucose levels. The doses of the leaf extract used in this study were found to be non-toxic as there were no significant changes after administration of the extract in normal rats.

Suganya et al[40] demonstrated that oral administration of aqueous extract of *Costus pictus* leaves (200 mg/Kg BW) to alloxan induced diabetic rats for 30 days showed significant reduction in blood glucose levels, urea, creatinine and increased the levels and activity of antioxidant enzymes. Administration of extract also resulted in significant increase in the concentration of total protein, albumin, globulin and A/G ratio. SGOT and SGPT levels were restored to normal levels after treatment with extract indicating restoration of normal liver functions. Also, reduced cholesterol and triglyceride levels were observed in the extract treated rats. The non toxic nature of the extract at the doses studied was confirmed with histopathological

studies of liver and pancreas which revealed a normal hepatic and pancreatic morphology.

Sethumathi et al[41] evaluated the antidiabetic and antioxidant activity of methanolic extracts of *Costus pictus* leaves administered orally as single daily dose of 120 mg/Kg BW and 180 mg/Kg BW in two different groups of alloxan induced diabetic rats. Tolbutamide (100 mg/Kg BW) was used as the standard. The results showed significant reduction in blood glucose levels and lipid peroxidation. Treatment with the extract also resulted in significant increase in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), vitamin A, vitamin C, vitamin E and reduced glutathione (GSH) levels. SOD and CAT are considered as primary enzymes involved in free radical scavenging. GPx in sufficient concentrations detoxifies H_2O_2. Vitamins A, C and E are powerful antioxidants by free radical scavenging or trapping. Reduction in TBARS levels of liver and kidney was noted in the extract treated diabetic rats. The reduced levels of total proteins in liver and kidney of the diabetic rats were restored to normal levels after treatment with the extract and these results were comparable with that of the standard drug. The author claimed that the methanolic extract of *Costus pictus* leaves possess free radical scavenging activity which was beneficial in restoring the pathological alterations caused by free radicals generated from lipid peroxidation process. The results were conclusive of the

anti-hyperglycemic effects and antioxidant property of methanolic extracts of *Costus pictus* leaves.

Sathuvan et al.[42] confirmed the *in vitro* antioxidant potential of different fractions of bark of *Costus pictus* D. Don using various assays (DPPH radical assay, superoxide anion radical scavenging assay, nitric oxide radical scavenging assay, ferrous metal ion chelating ability, hydrogen peroxide scavenging assay and total reducing power). The total phenol and flavanoid content of different fractions of the extract were also determined. A secondary metabolite profile was done by TLC and the active components were further analyzed using GC-MS method. Phenolic compounds and flavanoids which are considered to be major antioxidants of plant sources were found to be higher in the extract. Among the different fractions studied, the chloroform bark extract of *Costus pictus* was shown to possess highest antioxidant activity.

4.1.8.3 Antidiabetic activity

Jayasri et al[4] studied the antidiabetic effect of aqueous extract of *Costus pictus* leaves (2 g/Kg BW) administered orally for 28 days in normal and streptozotocin induced diabetic rats. The results showed a significant reduction in fasting blood glucose and increase in the serum insulin levels. There was also significant increase in body weight of rats treated with the extract. Other parameters which showed significant

reduction were TBARS, urea, albumin, SGOT, SGPT, triglycerides and total cholesterol. With administration of aqueous extract to diabetic rats there was a significant increase in their body weights. Phytochemical screening and trace element analysis of *Costus pictus* leaves were also done. The results revealed that *Costus pictus* leaves contain various phytochemicals like alkaloids, glycosides, carbohydrates, saponins, proteins and phenols and appreciable amounts of trace elements like Fe, Cu, Mn, Ca, K, Cr and Zn. The study attributed the presence of these trace elements to be responsible for potentiating insulin activity. The non toxic nature of the extract was also confirmed from the histopathology sections of liver and kidney which revealed a normal architecture. The results were conclusive of the antidiabetic activity of the *Costus pictus* aqueous leaf extract.

4.1.8.4 Hypolipidemic activity

A study by Remya et al.[5] revealed that oral administration of fresh leaf extract of *Costus pictus* (200 and 400 mg/Kg BW) significantly reduced plasma lipid levels (cholesterol, free fatty acid, phospholipids, triglycerides, LDL, VLDL) while increased HDL levels. The reduction seen in the lipid profile was higher at a dose of 400 mg/Kg BW when compared to 200 mg/Kg BW. The study confirmed the hypolipidemic effect of the extract and this effect was directly correlated to the decrease in blood glucose levels.

Jayasri et al.[4] in a study evaluating the antidiabetic effect of *Costus pictus* leaves in streptozotocin induced diabetic rats, has demonstrated that treatment with aqueous leaf solution of *Costus pictus*, caused significant decrease in levels of total cholesterol, triglycerides and lipids. The author attributed this effect on the lipid profile directly to the improved insulin levels as observed in this study.

4.1.8.5 Anthelmintic activity

Thomas et al[2] performed *in vitro* anthelmintic activity screening of hydro-alcoholic extracts of *Costus pictus* rhizomes against earthworms and compared with that of standard drug piperazine citrate at varying concentrations (0.5, 0.75 and 1 g%). It was shown in the study that the rhizome extract had significant anthelmintic activity. Time taken for complete paralysis after treatment with extract was comparable with the standard drug while there was significantly higher activity on death of worms when compared to that of the standard at the similar concentrations. A phytochemical evaluation of the hydroalcoholic extract of rhizomes was also carried out using a set of standard chemical tests as a part of this study. The results confirmed the presence of flavanoids, tannins, phenolic compounds carbohydrates, saponin glycosides and steroids. The study however did not show any correlation between the results of phytochemical constituents identified and the anthelmintic activity.

4.1.8.6 Antimicrobial activity

Majumdar et al[6] tested the *in vitro* antibacterial activity of methanol and aqueous extracts of *Costus pictus* leaf, root, stem and flower against *Escherischia coli, Shigella flexneri, Bacillus subtilis* and *Klebsiella pneumonia*, using agar well diffusion and broth dilution methods. A concentration of 150 μg/mL was used to determine the zone of inhibition and concentrations of 5-65 μg/mL was used to determine the minimum inhibitory concentration. The results proved that both aqueous and methanolic extracts of plant parts have inhibitory effect on the growth of the four tested microbes. The inhibitory effects were significant for *Escherischia coli, Shigella flexneri and Bacillus subtilis*. However, at the concentrations used it was seen that there was no significant inhibitory effect on *Klebsiella pneumonia*. The inhibitory effects of methanolic leaf extract were higher than the aqueous leaf extracts against these three bacteria. The methanolic extract of root showed maximum inhibition against *Shigella flexneri* (MIC - 30 μg/mL) while the flower extract was found to be highly sensitive against *Klebsiella pneumoniae* (MIC - 20 μg/mL). The antibacterial activity of the extracts was comparable with the standard antibiotics screened under similar laboratory conditions.

Reddy et al.[33] evaluated the antibacterial activity of leaf essential oil of *Costus pictus* (1%, 5% and 10% concentrations) against ten pathogenic bacteria namely *Staphylococcus aureus, Streptococcus*

faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella paratyphi, Enterobacter faecalis, Bacillus cereus, Proteus vulgaris and Serratia marcescens using agar diffusion method. The study confirmed significant but varying degrees of antibacterial activity on the tested microbes and the results were comparable with standard antibiotics (ofloxacin, ciprofloxacin, tobramycin and gentamicin sulphate) when screened under similar conditions. The major components of the leaf essential oil such as fatty acids and ionones were attributed by the author for its antibacterial activity.

Manjula et al.[43] evaluated the antibacterial property of leaves of *Costus pictus* D. Don. Heat Stable Proteins (HSP) and Total Soluble Proteins (TSP) analysis showed high HSP (0.9 mg/mL) and TSP (7.8 mg/mL) content in leaves. The HSPs of the leaves of *Costus pictus* were extracted and used for evaluation of antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas.* The leaf protein extract revealed significant inhibition on all the tested bacteria, exhibiting maximum activity at a concentration of 100 µL/L. The results proved that the leaf protein extract of *Costus pictus* possessed significant antibacterial activity.

Sulakshana et al.[7] evaluated the antibacterial activity of rhizome extracts (500-2000 µg) of three species of *Costus* (*Costus speciosus, Costus pictus and Costus igneus*) against gram positive bacteria

(*Bacillus subtilis, Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*). The antibacterial activity of the extracts was reported to be higher with increasing concentrations of the extract. The zone of inhibition diameter was found to be higher with *Costus pictus* extract against *Staphylococcus aureus* (1.26 cm), *Pseudomonas aeruginosa* (1.21 cm) and *Escherichia coli* (1.18cm). *Bacillus subtilis* showed the least zone of inhibition (1.12 cm). This study concluded that rhizome extracts of all three *Costus* species possessed potent antibacterial activity.

4.1.8.7 Anticancer activity

Sathuvan et al.[42] performed *in vitro* evaluation of different fractions (chloroform, methanol soluble and methanol insoluble) of bark of *Costus pictus* for its cytotoxic effect on HT29 (colon cancer) and A459 (lung carcinoma) cell lines using MTT assay. The trypan blue dye exclusion test was used to assess the cell viability. Treatment of cell lines with varying doses of the extract fractions (50 µg -250 µg for 24 hours) showed a significant dose-dependent reduction in the viability of HT29 and A549 cells. The total phenol and flavanoid content was also assessed in this study. The cytotoxic activity of the extracts against both the cell lines was attributed to the presence of flavanoids in these extracts.

Choudhury et al.[10] stuided the antiproliferative and apoptotic potential of *Costus pictus* on MOLT- 4 human cancer cell line (human

acute lymphoblastic leukemia) and normal cell line (human peripheral lymphocytes). Aqueous and alcoholic extracts at different concentrations were prepared from dried leaves of *Costus pictus*. Cytotoxicity was assessed by trypan blue dye exclusion test and MTT assay and the apoptic potential was analyzed by DNA fragmentation analysis of the treated cells. The ethanol extract of *Costus pictus* leaves was found to be highly cytotoxic (cell viability was less than 50% at 120 μg/mL concentration) when compared to the aqueous and methanol extracts. The results of the study reported that the cytotoxicity was concentration dependent. The ethanolic extract also activated the apoptotic pathway in MOLT- 4 cells. The same concentration was not cytotoxic to normal lymphocytes. This study confirmed the pro-apototic and anticancer potential of the ethanol fraction of *Costus pictus* leaves.

4.2 Oxidative stress

An imbalance between oxidant and antioxidant mechanisms in our body results in oxidative stress in which free radical production can react indiscriminately to cause damage to almost any of the cellular component including carbohydrates, lipids, proteins, and DNA.[17,18,22] Free radicals are continuously being produced endogenously as of part normal cellular functions or exogenously as a result of various environmental sources like pollutants, ultraviolet light, ionizing radiations, cigarette smoking, xenobiotics etc.[44] Oxygen derivatives (superoxide anion, hydroxyl radical

and hydrogen peroxide) and reactive nitrogen species (nitric oxide and peroxynitrite) are the most important free radicals involved in various disease states like cardiovascular, autoimmune, diabetes mellitus, cancers, infectious and inflammatory conditions and nervous system disorders.[19,44]

Antioxidants are the defense systems (endogenous or exogenous) which prevent free radical induced tissue damage by either preventing their formation, scavenging them, or by accelerating their degradation.[42,44] They can be categorized into enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin), transition metal binding proteins (tranferrin, ferritin, lactoferrin) and chain breaking antioxidants. The chain breaking antioxidants are further classified into lipid phase (tocopherols, carotenoids, flavonoids, ubiquinol) and aqueous phase (ascorbate, urate, glutathione and other thiols).[44] Phytomedicines are good sources of natural antioxidants like carotenoids, ascorbic acid, α-tocopherol, flavonoids, and phenolic compounds.[45]

4.2.1 Role of oxidative stress in pain

A study on 137 patients with acute and chronic inflammatory or non-inflammatory back pain by Inamr et al. found out that the oxidative stress levels (as indicated by malondialdehyde (MDA) levels) were significantly higher in patients when compared to controls. This study however did not show any correlation for the levels of oxidative stress with pain severity and pain threshold.[20]

A study by Taha et al. revealed the role of oxidative stress in the pathogenesis of complex regional pain syndrome (CPRS). Nuclear factor erythroid 2-related factor (Nrf2) which is an important regulator of transcription of antioxidants has been proved to have anti nociceptive effects against inflammatory pain in an animal model. This study has also correlated the protective role of Nrf2 in clinical progression of CPRS.[19]

Various studies suggest that oxidative stress may alter nociception by causing central and peripheral sensitization. Free radical production stimulates the sensory neurons and plays an important role in pain transmission.[20]

4.2.2 Role of oxidative stress in epilepsy

Brain is considered to be highly sensitive to oxidative damage.[21] Oxidative stress is an important underlying mechanism in the development and progression of various neurological diseases including epilepsy.[45] A study by Dalton et al. was the first to reveal that the severe brain damage caused by treatment of brain with kainic acid in a rat model of epilepsy, was partly caused by oxidative stress.[21]

Free radicals produced as a result of oxidative stress may induce seizure activity either by increasing the concentration of excitatory neurotransmitter glutamic acid (result of direct inactivation of glutamine synthase) or by a decrease in the brain cortex content of inhibitory

neurotransmitter GABA (result of inhibition of enzyme glutamate decarboxylase by oxygen free radicals).[45]

Seizure induced free radical damage and mitochondrial dysfunction results in redox alteration and can lead to an increased seizure susceptibility and further development of subsequent epilepsy.[21,22,45] In the brain, enzymatic and non enzymatic antioxidants play a significant role in repairing oxidative damage. Of which, glutathione is considered to be the major antioxidant defense system in the brain.[22]

Based on the evidence from animal models and clinical studies, anti oxidant therapies are recently being considered as an important therapeutic strategy in epilepsy,[21,46] including those which are refractory to treatment with conventional antiepileptic drugs.[21,47]

4.3 Methods of Screening for analgesic activity

Pain is an unpleasant symptom of almost every disease requiring appropriate and prompt treatment with analgesics (narcotics or non-narcotics) which act either by central or peripheral mechanisms without affecting consciousness.[11,48] Pain is a perception and a common phenomenon in all vertebrate animals and analgesic effects in animals are comparable to that in man.[49] Pain constitutes an alarm system to limit the potentially damaging consequences of the underlying factor which caused the pain. The term *nociception* was derived from the Latin *nocere*, meaning "to harm".[49] *Nociceptive* refers to a stimulus that is capable of

producing a tissue lesion and evokes a reaction from the organism.[49] "Algogenic" refers to the capacity of a stimulus to produce pain, which include sensory, affective and motivational components.[49] A "double pain" phenomenon is seen in humans after a brief nociceptive stimulus – "fast" pain (stinging and well localized) occuring as a result of activation of A δ fibres and "slow" pain (burning in nature, more intense, and more difficult to localize) which results from activation of C fibres and is slower in onset.[12,50]

Pain evaluation is difficult in animals because of the absence of verbal communication, particularly when the animal does not exhibit a typical physical sign or overt behaviours. Physical signs are considered the most reliable signs of pain. Reactions to pain include basic motor responses (withdrawal, jumping, contractures, etc.), reactions with an increase in sympathetic tone (mydriasis, hyperpnea, tachycardia, arterial hypertension etc), and vocalization. Complex reactions to pain like behavioural responses - escape, distrust of objects responsible for painful experiences, avoidance, aggressiveness, modifications of behaviour (social, food, sexual, sleep, etc.) and passive motor responses (immobility allowing the animal to preserve a painless posture) are also often observed in animals.[49]

Animal models have their own limitations. Preclinical animal models may not adequately predict the clinical efficacy in humans because species differences can significantly alter the responses to

pharmacological therapy.[50] The inter species variations can also lead to wrong doses due to the pharmacokinetic differences.[50] Large variations occur even in closely related species like that of rats and mice.[50] Analgesic screening models commonly used look predominantly on evoked pain (experimental pain under controlled conditions) which may significantly vary from the actual clinical pain as seen in humans.[50]

Figure 1: Models for evaluating analgesic activity[51-54]

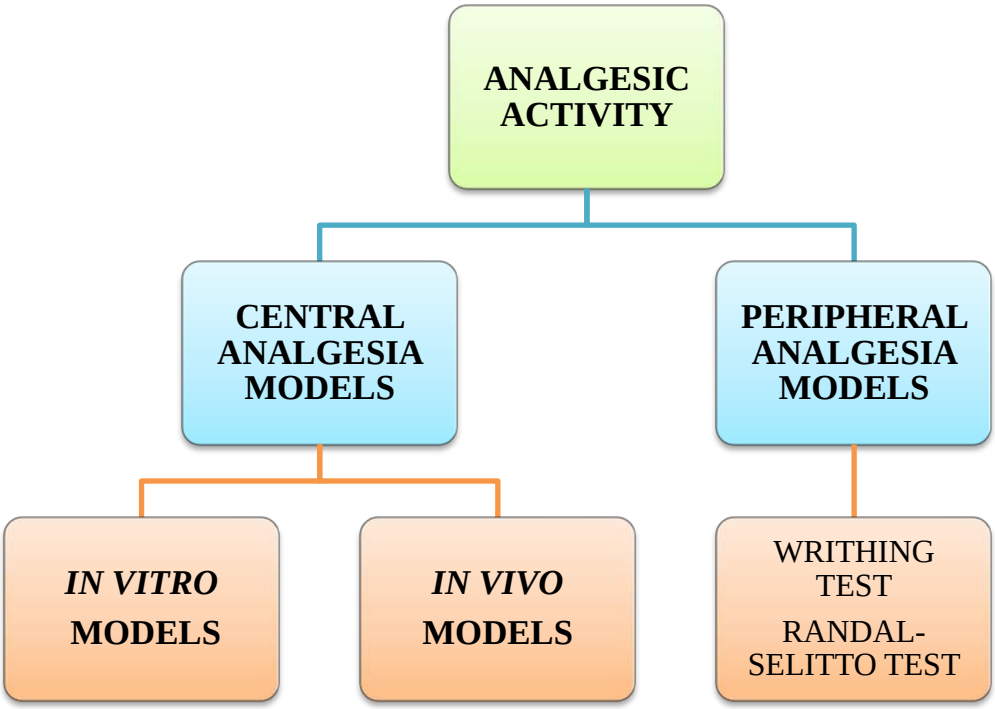
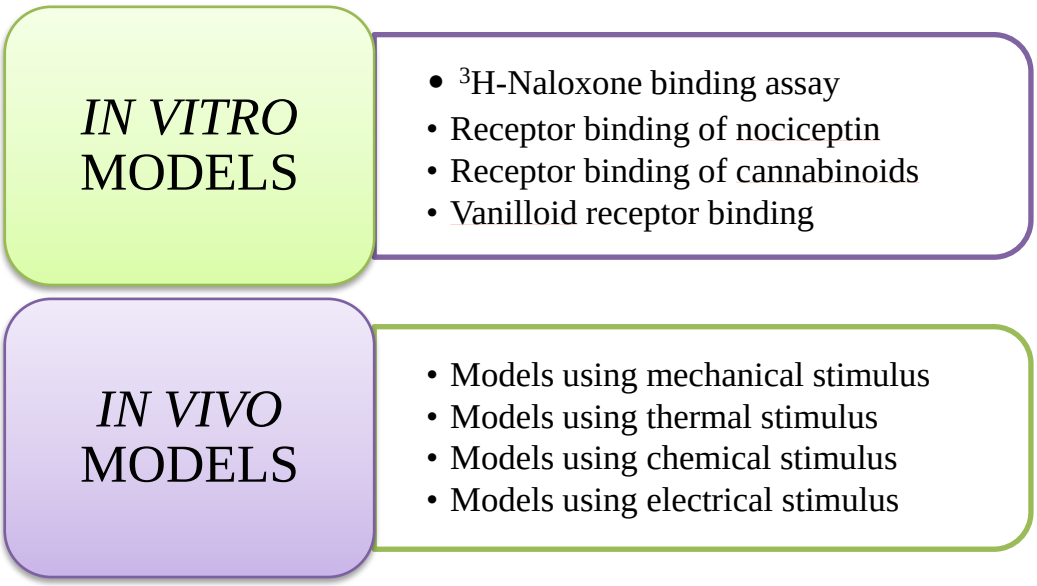


Figure 2: Models for evaluating central analgesic activity[51-54]



4.3.1 *In vivo* models for testing central analgesic activity[48,51-54]

Animal models are still essential in analgesic research. Rodents (mice or rats) are most commonly used while in certain instances higher animals like monkeys are used. Different types of nociceptive stimuli (electrical, mechanical, thermal and chemical) are used in various animal models.

4.3.1.1 Models using mechanical stimulus[48,51-54]

4.3.1.1.1 Haffner's tail clip method[48,51-54]

This is a very sensitive model for screening centrally acting analgesics. Peripheral analgesics cannot be detected by this model. Hind paw and tail are the usually the preferred sites for applying nociceptive mechanical stimulus. An artery clip applied to the root of the tail is commonly used to induce acute pain. The animal usually responds immediately by biting the clip or the tail near

which the clip is placed. Reaction time (time between clip application and response of the animal) is recorded using stop watch. Cut off time is calculated from the control group by taking the average reaction time plus three times the standard deviation of the combined latencies. The animals are observed till the cut off time. The cut off time of the test group is compared with that of the control and standard groups to determine whether there is any significant difference between the groups. If the reaction time of the test group is greater than the determined cut-off time, the test drug is said to have high analgesic effect.

4.3.1.2 Models using thermal stimulus[48,51-54]

In these models, only skin is stimulated without involvement of visceral or musculoskeletal tissues. The animal usually reacts by withdrawing itself quickly away from the thermal stimulus. These models are used to differentiate between the centrally acting opiate and non opiate analgesics. The response elicited in hot plate method is mostly at supraspinal level while the tail flick response is a predominant spinal response. These models are used to assess the threshold for response to a high intensity acute painful stimulus. Sedatives, psychotomimetics and muscle relaxants may produce false positive results by prolonging the reaction time.

4.3.1.2.1 Hot plate method[48,51-54]

It is a widely used model to evaluate centrally acting analgesics. The thermal stimulus is used to induce acute pain. Since the paws of rodents are very sensitive to heat at temperatures not damaging the skin, paws are preferred for the evaluation. Animals are placed on a hot plate, which consists of an electrically heated surface (made of copper, iron or aluminium) which is maintained by the thermostat knob at a temperature range of 55 °C to 56 °C. The animals immediately respond by jumping, licking or withdrawal of paws. The latency period (the time between onset of stimulus and response of the animal) is recorded using stopwatch and is compared between the control, standard and test groups. The cut off time for observation is 15 to 20 seconds for mice and 20 to 30 seconds for rat.

4.3.1.2.2 Tail-Flick test[48,51-53]

This model is a widely used and reliable test for evaluating opioid analgesics. Heat is used as noxious stimulus and the animal responds by flicking away its tail. The advantage of this model is that there is minimal inter animal variation. There are two variants of the tail-flick test.

a. Application of radiant heat on to a small surface of tail
b. Immersion of tail in water at a predetermined temperature

4.3.1.2.2.1 Radiant heat method[48,51-53]

This model measures the drug-induced changes in the sensitivity of mice or rats to heat stress applied to their tails. Animals are placed into restrainers with their tails exposed. A light beam focused (radiant heat application) on the proximal third of the tail or a hot wire serves as the noxious stimulus. The reaction of each animal is observed for about 6 seconds. Mice with a reaction time of more than 6 seconds are not used in the test. Within few seconds the animal flicks the tail aside and rotates head and tries to escape [escape reaction]. Reaction time (time between radiant heat application and escape reaction) is recorded using stop watch. Lengthening of reaction time indicates analgesic activity of the test drug. In order to avoid severe injury to the tail, the cut off time is set as 15 to 20 seconds for mouse and 20 to 30 seconds for rat.

4.3.1.2.2.2 Tail immersion method[48,51-54]

Centrally acting analgesics are evaluated by this method. Rats or mice are used for this test. Each animal is placed in separate cage such that the tail hangs freely. The distal 5 cm is marked which is immersed in a cup filled with water at a temperature of 55 °C for a maximum time of 15 seconds. Reaction time (time for tail withdrawal reflex) is observed with a stop watch. Normal time for response is 1-5.5 seconds. Drugs with central analgesic activity prolong the reaction time of the typical tail-withdrawal reflex in rats. Withdrawal time of

more than 6 seconds in test group animals indicates analgesic activity of the test drug.

4.3.1.3 Models using electrical stimulus[48,51,52,54]

The advantages of these models are that they are non-invasive and the results produced are quantifiable and reproducible. Well synchronized afferent signals are being produced. The disadvantages are that these stimuli are not a natural type of stimulus as encountered by animal in natural environment. Such stimuli excite all peripheral sensory fibres, which are not directly involved in pain. Thus, studies on peripheral transduction mechanisms are difficult with electrical stimulus. Variations can occur due to the differences in the impedances of tissues being stimulated.

4.3.1.3.1 Grid shock test[48,51,52,54]

This test measures the analgesic properties by "Flinch-jump" procedure. This method was first described in mice by Blake et al. Rats or mice are used. Electric stimulus is applied via plastic chambers. Stainless steel wires, spaced about 1 mm apart are tightly wired on to the floor of the box. Square wave pulses at a frequency of 30 cycles per second are given for duration of 2 milliseconds per pulse. The stimulator output is connected to alternate wires of the grid. A fixed resistance is placed in series with the grid and in parallel to an oscilloscope to allow calibration in milliamperes. Paws of the rodents are usually preferred for application of the stimulus.

Animals are placed individually in the plastic chamber and electrical stimuli of increasing intensities are applied and observed. With increasing shock intensities the animal will flinch, exhibit a startling reaction, locomotor activity increases or the animal attempts to jump. The behavior is recorded on an oscilloscope as marked fluctuations of the displayed pulse. Pain thresholds are determined for each individual animal. The current as measured in milliamperes is recorded for each animal before and after administration of the drug.

Variations of this method:

- **Fractional escape procedure -** animals are trained to press a lever to reduce the intensity of electrical stimulus delivered continuously through the floor grids of the experimental chamber. An external timer increases the intensity of the shock every few seconds. If the animal fails to press the lever, the shock continues to increase. Analgesic property of a drug is measured as the alteration in the level of shock which the rat will "tolerate".
- **Pododolorimetry -** Painful stimulation of the paws of mice placed into cages equipped with metal bands
- An automated method to analyze vocalization of unrestrained rats after noxious stimuli.

4.3.1.3.2 Electrical stimulation of tail[48,51,52,54]

Tail of mice is sensitive to any kind of stimulus. Electrical stimulation model has been first described by Burn et al. Variations in the duration or intensity of the electrical stimulus are used to observe the response. The effect of central analgesics and also the activity of peripheral analgesics at higher doses can be clearly demonstrated by this method. Mice are placed in special cages. A pair of alligator clips is placed on the tail. A positive electrode is placed on the tail at the proximal end. Rectangular wave pulses with an intensity of 40–50 V and frequency of 1 shock per second are delivered from a constant voltage stimulator for duration of 2.5 milliseconds. Animal normally responds to the stimuli in 3 to 4 seconds. Following drug administration, the response time is observed and recorded every 15 minutes, until the reaction time returns to control levels.

Ultrasonic stimulation may be used instead of electrical stimulus. The advantage of this method is that it is fast, simple, and precise. Repeated stimuli can be used without causing tissue damage.

4.3.1.3.3 Tooth pulp stimulation test[48,51,52,54]

This method was first described by Kohl and Reffert (1938) and by Ruckstuhl and Gordanoff (1939) for testing central analgesic activity in rabbits, which was later adopted in several other species. This method is found to be highly sensitive for central analgesics,

especially opioids. Non-opiate analgesics like ketamine and peripheral analgesics like pyrazolone derivatives also show positive response in this method. Characteristic reactions such as licking, biting, chewing and head flick which can be easily observed are induced by tooth pulp stimulation.

Rabbits are commonly used. Animals are anaesthetized with thiopental (15 mg/Kg) or fentanyl citrate (0.2 mg/Kg) intravenously. Pulp chambers are drilled close to the gingival line in the lateral margins of the two front upper incisors using a high-speed dental drill. Before the experiment, the clamping electrodes are placed into the drilled pulp chambers. After thirty minutes, a rectangular current with a frequency of 50 Hz is given for duration of 1 second. The electrical stimulus is started with 0.2 milliamperes and increased until licking occurs to determine the threshold current. Threshold is determined at various pre determined time intervals with respect to drug administration.

The intensity of threshold current, expressed in mV is used as an indicator of the intensity and duration of analgesic effect. Those drugs which produce an increase of the threshold versus the initial control by a factor of 2 or more is considered to possess anti nociceptive effect.

Dogs and cats have been used instead of rabbits. Microinfusion of bradykinin solution onto the tooth pulp of unrestrained rats is

used as a reliable method for evaluating analgesic effect of drugs on trigeminal pain.

4.3.1.3.4 Monkey shock titration test[48,51,52,54]

Analgesic tests in rodents (mice and rats) are closely correlated with analgesic activity of a drug in humans. Tests in higher animals like monkeys are necessary to clarify mode of action of test drug and to determine a suitable human dose. This model is not recommended for routine screening.

Monkeys are restrained in chairs and an electrical current is delivered by a Coulbourn Instrument Programmable Shocker through electrodes coupled to two test tube clamps. These electrodes are attached to a shaved portion of the monkey's tail. The intensity of electrical stimulus is increased from 0 to 4 mA through 29 progressive steps and the monkey presses a bar to interrupt the shock. A stable baseline shock level is determined for each monkey on the day prior to drug administration. After administration of test drug, the change in maximum level of median shock intensity attained for drug is compared to control levels. Monkey presses a bar to interrupt the shock. This is considered to the response. The shock intensity at which the animal produces the response is noted. The change in maximum level of median shock intensity for test drug is compared to control levels.

4.3.1.4 Models using chemical stimulus[48,51,52,54]

Administration of algogenic agents represents a slow form of stimulation. Chemical stimulation is progressive and of longer duration, and once applied inescapable. These models do not measure the threshold but behavioral scores and are closest to clinical pain.

4.3.1.4.1 Formalin test in rats[48,51,52,54]

This model is used as a chronic pain model which is sensitive to centrally active analgesic agents, whereas peripherally acting analgesics are almost ineffective. This method is useful to differentiate centrally acting morphine-like analgesics and non-opiate analgesics and allows discrimination between inflammatory and non-inflammatory pain.

Wistar albino rats are commonly used. 0.05 mL of 10% formalin is injected into the dorsal portion of the front paw. The test drug is administered simultaneously either orally or subcutaneously. Each rat is placed individually into a clear plastic cage and observed. Scoring according to a pain scale is done at 30 and 60 minutes. Elevation or favouring of the paw or excessive licking and biting of the paw is considered as the response. Analgesic property of the test drug is indicated if both the paws are resting on the floor with no obvious favouring of the injected paw.

4.3.2 *In vivo* models for testing peripheral analgesic activity[48,51,52,54]

Peripheral analgesics exhibit anti-inflammatory and antipyretic activity besides analgesia. The most commonly used methods for evaluating peripheral analgesic activity are the writhing tests in mice and the Randall-selitto test in rats.

4.3.2.1 Writhing tests[48,51,52,54]

Pain is induced by injection of irritants like phenylquinone or acetic acid into the peritoneal cavity of mice. The animals react with a characteristic stretching behavior of abdomen with simultaneous stretching of atleast one hind limb which is called writhing**.** This model can be used to detect both central and peripheral analgesic activity. It is considered as a simple screening method. The limitation of this method is that it lacks specificity. Some psychoactive agents like haloperidol also show activity in this model.

Mice are commonly used. 0.25 mL of phenylquinone (0.02%) suspended in 1% carboxymethylcellulose is injected into the peritoneal cavity of mice. Each animal after drug administration is placed individually into glass beakers and after five minutes they are observed for a period of ten minutes and the number of writhes is recorded for each animal. Average number of writhes in each group and percent inhibition are calculated and recorded. Percent inhibition is calculated by the formula given below:

$$\frac{\text{Average writhes (Control) – Average writhes (Test)}}{\text{Average writhes (Control)}} \times 100$$

If the value of the calculated percent inhibition is more than 70 %, the drug at that particular dose is considered to have significant analgesic activity.

Intraperitoneal injections of other inducing agents used are acetylcholine, prostaglandin E1, aconitine, 4% sodium chloride solution, 7 mL air or 6% aqueous saline and ethacrinic acid.

4.3.2.2 Randall-selitto test[48,51,52,54]

This model is based on the principle that inflammation increases pain sensitivity which can be modified by analgesics. Inflammation decreases the threshold to pain while this threshold can be increased by non-narcotic analgesics and narcotic analgesics. Brewer's yeast can be used to induce inflammation which increases pain after pressure.

Wistar albino rats are used. Subcutaneous injection of 0.1 mL of a 20% suspension of Brewer's yeast in distilled water into the plantar surface of the hind paw of the rat is used to induce inflammation. Three hours later, a special apparatus is used to apply pressure / force (measured in grams) at a constant rate to the plantar surface of rat's foot till the animal struggles, squeals or attempts to bite. Each animal is tested individually for its control pain threshold and animals with threshold lesser than 80 g are included in the test. The tests are done at intervals of 15 minutes after subcutaneous administration and at

intervals of 30 minutes after oral administration for any change in pain threshold. The interval of time at which the increase in pain threshold is highest is considered as the peak time. The test drug is administered in a random manner. The pain threshold for the test drug is recorded at time zero and again at the pre-determined peak time (control value).

The mean applied force is determined for each time interval. Percentage increase in pain threshold by the test drug is calculated as shown below:

$$\frac{\text{Applied force (test drug) - Applied force (Control)}}{\text{Applied force (Control)}}$$

This model can detect both central and peripheral analgesics. Peripherally acting analgesics increase only the threshold of the inflamed paw, whereas opiate analgesics increase the threshold of the intact paw also. Commercially available Randall-Selitto analgesy meters can also be used.

4.4 Methods of Screening for anticonvulsant activity

Epilepsy is a chronic neurological disorder of disturbed electrical activity of the brain.[14] It is widely prevalent throughout the world and associated with significant morbidity.[55] Epilepsy is a collection of different types of seizures. Prolonged and repetitive attacks significantly affect the quality of life and may be life threatening.[14] They are often associated with depression, anxiety, neurodevelopmental delay, impairment of cognition and memory. Often these comorbidities are left unaddressed, and the treatment is usually

focused on seizure control alone.[16] Epileptogenesis involves multiple heterogenous mechanisms.[14,16] Epilpesy can occur due to alterations in voltage-dependent ion channels resulting either in decrease in inhibitory (GABA-mediated) inputs or increase in excitatory (glutamate mediated) inputs.[15,16]

Despite appropriate and adequate treatment with currently available antiepileptic drugs (AEDs), 25-40% patients are resistant to pharmacotherapy.[55] Though monotherapy may be effective in 50-70% of patients, seizure freedom is often achieved with combination therapy of anti epileptic drugs with different mechanisms of action.[16] Complex pharmacokinetic interactions and drug induced adverse effects are important concerns with current antiepileptic therapy.[15,16]

Preclinical screening using animal models have played a crucial role in the discovery of various clinically effective AEDs. Most currently used models represent epileptic seizures rather than epilepsy as such.[55,56] The currently used animal models for epilepsy do not elucidate the mechanisms underlying preictal, interictal, ictal and postictal states. These are significant phases associated with behavioral changes in humans. Newer animal models are needed to address these limitations.[56]

4.4.1 Methods of screening antiepileptic drugs[57,58]

In vitro methods are –

- Electrical recording from isolated brain cells
- *In vitro* assays for GABA receptor binding

- GABA uptake in rat cerebral cortex synaptosomes
- GABA uptake and release in rat hippocampal slices
- Excitatory amino acid (glutamate, glycine) receptor binding assays

In vivo methods are –

- Electrically induced seizure models
- Chemically induced seizure models
- Focal lesions induced seizure models
- Models of status epilepticus
- Models of infantile spasms
- Genetic models of epilepsy

4.4.2 Characteristics of an ideal model of epilepsy[55]

None of the currently used animal model represents an ideal epilepsy model. An ideal animal model of epilepsy should reflect a similar pathophysiology as seen in humans and should also provide scope to assess the efficacy of the drug against pharmacoresistance. The ability of a drug to modify progression of epilepsy following an insult should also be assessed. Following factors should be considered for an ideal animal model of human epilepsy:

- Pathological findings consistent with human epilepsy (e.g., cell loss, gliosis, neurogenesis, axonal and synaptic reorganization)
- Latent period following initial insult which is species-appropriate

- Spontaneous seizures following latent period
- Resistance seen with one or more antiepileptic drugs
- Associated co-morbidities like progressive neurobehavioral and cognitive impairment

4.4.3 *In vivo* models[53,57,58]

4.4.3.1 Electrically induced seizure models[53,57,58]

These models are used to screen drugs which are effective for generalized tonic-clonic (grandmal) and focal seizures where as anti-absence seizure drugs cannot be tested. Certain other centrally acting drugs also suppress the tonic hind limb extensions which are evoked by the electrical stimulus.

4.4.3.1.1 Maximal Electroshock induced Seizure (MES) model[53,57,58]

Rats or mice are commonly used. A stimulating apparatus with corneal or ear electrodes supplying a constant current [50 mA for mice and 150 mA for rats] at a frequency of 50-60 Hz is applied for a duration of 0.2 seconds. A constant voltage of 250V (mice) or 750V (rats) is used. The animals are observed for a period of 2 minutes after application of electrical stimulus. The seizure which occurs passes through various phases: phase of tonic limb flexion, phase of tonic limb extension and a variable short clonic interval. Efficacy of new antiepileptic drugs is measured by the suppression of tonic hind limb extension.

The limitations of this model are that though the eyes of the animals are moistened with a local anaesthetic before application of corneal electrodes, it has the risk of causing blindness. The variable short clonic interval may also cause asphyxial death in some animals.

4.4.3.1.2 Threshold models[57,58]

When used along with MES test, this model is useful to screen drugs which are effective against generalized tonic clonic seizures (GTCS). The drugs used in GTCS usually increase the seizure threshold. The drugs which increase the seizure threshold for tonic hind limb extension are considered to be effective.

Mice or rats are commonly used for this experiment. Electrical stimulation is given by placing corneal or ear electrodes which delivers a constant current or voltage at a frequency of 50-60 Hz for 0.2 seconds duration. Threshold is determined as the current or voltage which induces hind limb extension in 50% of the animals. Elevation of seizure threshold is taken as the measure of efficacy of the test drug.

4.4.3.1.3 Kindling models[57,58]

This is a model to develop seizures which involves delivery of repeated submaximal electrical or chemical stimulus. Progressive intensification of the stimulus, lowers the seizure threshold and results in a generalized seizure. Kindling demonstrates the fact that ‘epilepsy

induces epilepsy'. Amygdala is the most commonly chosen region for kindling. The advantage of this model is that the efficacy of the drug can be measured against epileptogenesis as well as the fully kindled state.

Adult Sprague-Dawley rats are used for this experiment. Electrical stimulation is done through an electrode placed in the right amygdala. After a recovery period of 1-2 weeks, daily electrical stimulus (400-500 μA, 1 millisecond monophasic square wave pulses for 1 sec at a frequency of 50-60/second) is applied through the electrode. Daily electrical stimulation results in seizures, which evolve through 5 stages:

Class 1 – immobility, eye closure, twitching of vibrissae, stereotypic sniffing

Class 2 – Facial clonus and head nodding

Class 3 - Facial clonus, head nodding and forelimb clonus

Class 4 – Rearing, often accompanied by bilateral forelimb clonus

Class 5 – Rearing with loss of balance and falling accompanied by generalized clonic seizures

Rats are considered fully kindled when class 5 seizures have developed. If stimulation continues for few weeks, rats develop spontaneous epileptic seizures. Four different measures for drug latency (seizure latency, seizure severity, seizure duration and after discharge duration) are recorded.

4.4.3.2 Chemically induced seizure models[53,57,58]

a. **Chemoconvulsants inducing generalized seizures after systemic administration -** Pentylenetetrazole, bicuculline, picrotoxin, penicillin, isoniazid, thiosemicarbazide, allylglycine, strychnine, pilocarpine, N-methyl D aspartate, kainic acid, gamma hydroxybutyric acid, etc

b. **Chemoconvulsants inducing focal seizures after central administration -** Penicillin, kainic acid, quinolinic acid, pentylenetetrazole

4.4.3.2.1 Pentylenetetrazole (PTZ) test[53,57,58]

PTZ is a tetrazole derivative having a consistent effect in a large number of animal species. Its action is through inhibition of GABA neurotransmission. It is a useful and commonly used model for screening drugs effective against petitmal epilepsy or absence seizures.

Subcutaneous PTZ (scPTZ) test - Drugs effective in petit mal epilepsy like ethosuximide and valproic acid are effective in this model of epilepsy, while phenytoin and carbamazepine are ineffective.

Rats or mice are used. CD_{97} of PTZ (Convulsive dose in 97% of the animals) is 70 mg/Kg in rats and 80-100 mg/Kg in mice. Control animals develop seizures within 30 minutes in a sequence of

excitement, myoclonic jerks, clonic seizures, one or more maximal clonic seizures and death. The first episode of clonic jerking lasting for 5 sec or the first clonic seizure with loss of righting reflex is taken as the end point.

4.4.3.3 Focal lesions induced seizure models[57,58]

- Seizures are induced with cortically implanted metals like alumina cream / gel, cobalt, tungstic acid or injection of iron into the brain cortex. Topical aluminium hydroxide gel model is most commonly used.
- Miscellaneous chemicals which are used to produce focal lesions are - intrahippocampal injections of kainic acid, topical applications of penicillin, cholinergics, picrotoxin, bicuculline, strychnine, zinc etc.
- Systemic focal epipleptogenesis (model combining features of focal and generalized epilepsy)

4.4.3.4 Models of Status Epilepticus[57,58]

- Pilocarpine induced status epilepticus
- Lithium-pilocarpine induced status epilepticus
- Lithium-methomyl induced seizures in rats
- Electrical stimulation of hippocampal perforant pathway
- D, L- homocysteine induced status epilepticus
- Generalized myoclonic seizures in baboons

4.4.3.5 Genetic models[57,58]

- Photosensitive baboons
- Seizure prone mice strains
  - Audiogenic seizure susceptible mice
  - Totterer mice
  - E1 mice
  - Quaking mice
  - Lethargic mice
- Seizure prone rat strains
  - Genetically epilepsy prone rats
  - Rats with spontaneously occurring petitmal epilepsy
- Mongolian gerbils
- Miscellaneous genetically prone animals

4.4.4 Widely used models in antiepileptic drug discovery

MES and PTZ (scPTZ) seizure models are widely used in screening newer agents for their antiepileptic property. These are preferred because of the convenience in conducting the experiment, potential to screen large number of drugs, lesser investment and minimal expertise. MES model is considered to represent human generalized tonic-clonic seizures and scPTZ model is sensitive to screen AEDs for generalized absence seizures.[55] MES model is not effective to screen drugs against partial seizures.[59] MES and PTZ models though useful in initial screening of drugs with antiepileptic activity represent models of acute (reactive or

provoked) seizures rather than models for epilepsy.[59] These models use non-epileptic animals. Acute seizure models may also be not useful in identification of drugs for pharmacoresistant seizures.[59] MES model has also failed to prove efficacy of several novel AEDs like levetiracetam, tiagabine and vigabatrin.[60] Though there are limitations, initial screening using simple animal models was crucial in the identification and development of all first and second generation AEDs except bromides and phenobarbital.[61]

For drugs targeting prevention and modification of epileptogenesis, chronic seizure models like kindling and post-status models (pilocarpine or kainate models) are widely useful.[61] Kindling is the only chronic model which has adequately predicted the clinical usefulness of novel AEDs againt partial seizures.[60] Chronic models use epileptic animals which have been chronically induced electrically or chemically and those animals with inborn epilepsy. Though chronic models are labor intensive, they give more predictive data when compared to acute seizure models.[59]

MATERIALS & METHODS

5. Material and Methods

5.1. Study design

Experimental *in vivo* study in animals

5.2. Study setting and duration

The study was conducted over a time period of six months (December 2014 – May 2015) at the Research laboratory, Department of Pharmacology, Sree Mookambika Institute of Medical Sciences [SMIMS], Kulasekharam, Kanyakumari District, Tamilnadu.

5.3. Ethics committee approval

The study was approved by the Institutional Animal Ethics Committee with reference number SMIMS/IAEC/2014/C/01 [dated 29/09/2014].

5.4. Animals used

5.4.1. Species

Healthy adult Wistar albino rats of either sex, weighing between 180-250 g were selected for the study. A total of 78 animals were obtained from the central animal house, Sree Mookambika Institute of Medical Sciences [SMIMS], Kulasekharam.

5.4.2. Housing conditions[62]

Adult Wistar albino rats of either sex were maintained in the central animal house of this institute as per the Committee for the

Purpose of Control and Supervision of Experiment on Animals (CPCSEA) guidelines. Male and female rats were housed in separate cages. Pregnancy was excluded among the female rats before selection for the study. All the animals which were used for this study were fed with standard commercial rodent diet and water *ad libitum.* The animals were transferred to the research laboratory one week before the experiment for acclimatization. Three rats were placed in a single plastic cage at room temperature (27±2 °C), humidity 70-80%, and 12 hour light and dark cycle was maintained. All experiments were conducted between 10:00 AM and 4:00 PM to minimize the variations in the data obtained.

5.5. Drugs and chemicals used

A. Vehicle control – Sterile water

B. Test drug – Hydroalcoholic extract of dried *Costus pictus* leaves. Doses used were 200 mg/Kg and 400 mg/Kg body weight. The doses were selected based on the previous studies done with *Costus pictus* leaves.[5,40]

C. Standard drugs[51,52.57,58]

- **For evaluating central analgesic activity -** Morphine (Morphine sulfate – 10 mg/mL; MORPHITROY 10; Troikaa Pharmaceuticals Ltd., Gujarat, India)

- **For evaluating peripheral analgesic activity -** Diclofenac (Diclofenac sodium – 75 mg/mL; DICSOL-AQUA; Sunvet Healthcare, Himachal Pradesh, India)
- **For MES model -** Diphenylhydantoin (Phenytoin sodium – 50 mg/mL; EPTOIN; Akums Drugs and Pharmaceuticals Ltd., Haridwar, India)
- **For PTZ model -** Sodium Valproate (Valproic acid – 100 mg/mL; ENCORATE; Unimed Technologies Ltd., Gujarat, India**)**

D. Drugs used to induce writhing[51]

- **4% Sodium chloride –** Sodium chloride salt for laboratory purpose (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India)

E. Drugs used to induce convulsions[57,58]

- **Pentylenetetrazole (PTZ) – (**Sigma Aldrich, USA)

F. Other drugs[57,58]

- **Lignocaine** (Lignocaine Hydrochloride injection; LOX 2%; VHB Medi Sciences Limited, Uttarakhand, India)

5.6. Equipments used

Artery clip, Eddy's hotplate (Guna Enterprises, Chennai, India), electroconvulsiometer (MSK Private Ltd. Chennai, India), stop watch, beakers, stirrers, spatula, china dish, conical flask, water bath, fine muslin

filter cloth, autoclave, refrigerator, airtight sterile container, electronic weighing machine, syringes (1, 3, 5 mL), oral feeding needle.

5.7. Preparation of leaf extract

5.7.1. Identification and authentication of *Costus pictus* leaves

Costus pictus was identified and its leaves were collected during the month of November 2014, from a home garden at Nagercoil, Kanyakumari district, Tamilnadu.

The *Costus pictus* leaves were authenticated by Botanist, Government Higher Secondary School, Karungulam, Thoothukudi District. The authenticated plant leaf was then kept as a specimen in the Museum, Department of Pharmacology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Kanyakumari district, Tamilnadu.

5.7.2. Preparation of dry leaf powder[31,63]

The collected *Costus pictus* leaves were washed with tap water and shade dried. The dried leaves were crushed into fine powder by mixer grinder.

5.7.3. Preparation of solvent

50% hydroalcohol solution was prepared by mixing absolute alcohol and water (50:50, v/v) and 1000 mL of 50% hydroalcohol solution was prepared.

5.7.4. Preparation of hydroalcoholic extract of *Costus pictus* leaves

The hydro-alcoholic extract was prepared by cold maceration method.[63,64] Dried powder was weighed using electronic weighing balance. 100 g of the dried leaf powder was taken and it was mixed with 750 mL of hydroalcohol solution in a conical flask. The solution was stirred well so that no clumps were formed. The solution thus prepared was kept for one week with intermittent stirring. On 8th day, the solution was boiled at 50 °C for 3 hours on a water bath. The solution was allowed to cool and was then filtered by using a fine muslin cloth. The powder which remained in the muslin cloth was again transferred into a conical flask and then mixed with 50% hydroalcohol solution and was used for the preparation of second batch of extract using the same procedure as mentioned above. The filtrate obtained was boiled at 90 °C on water bath till the solvent got evaporated. A semi solid dark brown extract was obtained after condensation. The two batches of condensed extracts were mixed together and the whole extract was weighed. The obtained extract was heat sterilized in an autoclave at 121 °C for thirty minutes.[65] The extract was transferred to an airtight sterile container and was refrigerated for further use in the study. Required concentrations of the extract were freshly prepared just before the experiment by weighing appropriate amount of the stored extract and diluting it with sterile distilled water.

5.7.4.1. Yield

For 100 g of dry leaf powder, 27.55 g of the condensed extract was obtained. (Yield = 27.55%)

5.8. Study procedure

The experiments were carried out as per the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). An acclimatization period of one week was given before starting the study. All the animals were handled with utmost care to minimize the stress and pain. The animals used for the experiments were fasted overnight with free access only to water. They were properly grouped and marked to avoid mixing of groups.

5.8.1. Grouping of animals

The animals used for the study were divided into thirteen groups [Group I to Group XIII], each consisting of six animals. A total of 78 animals were used for this study. Of which, 30 animals were used for evaluating analgesic activity and 48 animals were used to evaluate anticonvulsant activity. Same animals [Groups I to IV] were used to evaluate analgesic activity sequentially using different models (Haffner's tail clip method, hot plate method and writhing test) to reduce the number of animals used in the study. For writhing test, an additional positive control group [Group V] was used.

Table 1. Grouping of animals for evaluating analgesic activity[51-53,66]

Analgesic Activity	
Group I	Sterile water equivalent volume orally [Vehicle control for tail clip method, hot plate method and writhing test]
Group II	Morphine 5 mg/Kg BW intraperitoneally [Positive control for tail clip method, hot plate method and writhing test]
Group III	*Costus pictus* hydroalcoholic extract 200 mg/Kg BW orally [For tail clip method, hot plate method and writhing test]
Group IV	*Costus pictus* hydroalcoholic extract 400 mg/Kg BW orally [For tail clip method, hot plate method and writhing test]
Group V	Diclofenac sodium 12.5 mg/Kg BW orally [Positive control for writhing test]

n=6 in each group; BW – Body Weight;

Table 2. Grouping of animals for evaluating anticonvulsant activity[57,58]

Electrically induced convulsions (With current 150 mA, at frequency 60 Hz, for duration 0.2 seconds)	
Group VI	Sterile water equivalent volume orally [Vehicle control for MES model]
Group VII	Phenytoin 10 mg/Kg BW intraperitoneally [Positive control for MES model]
Group VIII	*Costus pictus* hydroalcoholic extract 200 mg/Kg BW orally [For MES model]
Group IX	*Costus pictus* hydroalcoholic extract 400 mg/Kg BW orally [For MES model]
Pentylenetetrazole (70 mg/Kg BW, s.c) induced convulsions	
Group X	Sterile water equivalent volume orally [Vehicle control for PTZ model]
Group XI	Sodium valproate 400 mg/Kg BW intraperitoneally [Positive control for PTZ model]
Group XII	*Costus pictus* hydroalcoholic extract 200 mg/Kg BW orally [For PTZ model]
Group XIII	*Costus pictus* hydroalcoholic extract 400 mg/Kg BW orally [For PTZ model]

n=6 in each group
BW – Body Weight; s.c – subcutaneous; mA – Milliamperes; Hz - Hertz
MES – Maximal Elecroshock induced Seizures; PTZ – Pentylenetetrazole

5.8.2. Evaluation of analgesic activity[51-53]

A total of five groups each consisting of six animals were used in the evaluation of analgesic activity. Each animal from groups I to group IV was individually tested in a sequential manner using different analgesic models. Haffner's Tail clip method and hot plate method were done first to evaluate the central analgesic activity. The same animals were then subsequently subjected to writhing test for testing peripheral analgesic activity. For writhing test, an additional group (Group V) was used. Animals in group V received diclofenac sodium as the standard drug.

5.8.2.1. Central analgesic activity[51-53]

5.8.2.1.1. Haffner's tail clip method[51-53]

Procedure: An artery clip was applied to the root of the rat's tail (approximately 1 cm from the body) to induce acute pain. The vehicle control (Sterile water – equivalent volume) and the test drugs (hydroalcoholic extract of *Costus pictus* – 200 mg/Kg BW and 400 mg/Kg BW) were administered orally through oral feeding needles to the animals of the corresponding groups as shown in Table 1. After 60 minutes of oral drug administration, each animal was tested individually by applying the tail clip. Animals of the standard drug group received morphine (5 mg/Kg body weight) intraperitoneally and 30 minutes later they were tested individually for the response.

Response: The animal normally responds to the noxious mechanical stimulus immediately within few seconds by biting the clip or the tail near the location of the clip.

Cut off time: The animals were observed for response only till the cut off time. It was calculated using the mean reaction time of control group plus three times its standard deviation.[54]

Evaluation: The latency to react (time between stimulation onset and response) in seconds, was observed using stop watch and noted.

5.8.2.1.2. Hot plate method[51-53]

Procedure: Commercially available hot plate consisting of an electrically heated surface maintained at a temperature of 55 °C to 56 °C, was used in this method. The vehicle control (sterile water – equivalent volume) and the test drugs (hydroalcoholic extract of *Costus pictus* – 200 mg/Kg BW and 400 mg/Kg BW) were administered orally to the animals of the corresponding groups as shown in Table 1. After 60 minutes of drug administration, each animal was placed individually over the preheated hot plate. Animals of the standard drug group were given morphine (5 mg/Kg BW) intraperitoneally and 30 minutes later tested for response. The animals were observed for response till the cut-off time.

Response: The animal usually responds to the heat stimulus by jumping, withdrawal of paws and licking of the paws.

Cut off time: The cut off time taken was 30 seconds.

Evaluation: The time until the above mentioned responses occur (reaction time) was noted in seconds, using a stopwatch.

5.8.2.2. Peripheral analgesic activity[51,52,66]

5.8.2.2.1. Writhing test[51,52,66]

Procedure: Standard (Morphine - 5 mg/Kg BW; intraperitoneally and Diclofenac sodium -12.5mg/Kg BW; orally) and test drugs (hydroalcoholic extract of *Costus pictus* – 200 mg/Kg BW and 400 mg/Kg BW) were administered orally to animals of the corresponding groups as shown in Table 1. Thirty minutes after intraperitoneal drug administration and sixty minutes after oral drug administration, writhing was induced with 4% NaCl (1 mL/Kg) which was injected intraperitoneally. Each rat was individually observed for the writhing response.

Response: Writhing response (indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb).

Evaluation: The time for onset of writhes and the number of writhes in each animal was observed for a period of 30 minutes. Percent inhibition was calculated by the formula given below:[51]

$$\frac{\text{Average writhes (Control)} - \text{Average writhes (Test)}}{\text{Average writhes (Control)}} \times 100$$

5.8.3. Evaluation of anticonvulsant activity[57,58]

5.8.3.1. MES model[57,58]

Procedure: The grouping of animals is shown in Table 2. The test drug (hydroalcoholic extract of *Costus pictus* – 200 mg/Kg BW and 400 mg/Kg BW) was given orally and standard drug (diphenylhydantoin – 10 mg/Kg) was injected intraperitoneally (i.p). 30 minutes after i.p injection and 60 minutes after oral administration, corneal electrodes were used to induce seizures. Before placing the electrodes, the eyes of the rats were moistened and anesthetized with lignocaine (2% solution). The electroconvulsiometer was set to deliver a stimulus of 150 mA intensity with a frequency of 60 Hz for duration of 0.2 seconds.

Evaluation: Tonic hind limb extension (present/absent) and seizure activity scoring were observed for each animal.

5.8.3.2. PTZ model[57,58]

Procedure: The grouping of animals is shown in Table 2. Each animal was weighed individually and the weights of the animals were noted. The test drugs (hydroalcoholic extract of *Costus pictus* – 200 mg/Kg and 400 mg/Kg) were given orally and standard drug (Sodium valproate – 400 mg/Kg) was injected intraperitoneally (i.p). 30 minutes after i.p injection and 60 minutes after oral administration, pentylenetetrazole was given in a dose of 70 mg/Kg subcutaneously. Each animal was then placed separately and

observed for one hour. Seizures and tonic convulsions were recorded. For each animal, time for onset of seizure, total number of seizures in one hour and duration of each seizure were noted. The total duration of seizure was obtained by adding up the duration of each seizure for an animal. The severity of seizures was also scored for each animal as mentioned below.

5.8.4. Parameters

A. <u>ANALGESIC ACTIVITY:</u>[51-53]

i. Haffner's tail clip

- Reaction time in seconds (time between onset of stimulus and response)

ii. Hot plate method

- Reaction time in seconds (time between onset of stimulus and response)

iii. Writhing test (Writhing is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb)

- Number of writhes in 30 minutes
- Percent inhibition was calculated by the formula given below[51]

$$\frac{\text{Average writhes (Control)} - \text{Average writhes (Test)}}{\text{Average writhes (Control)}} \times 100$$

B. ANTICONVULSANT ACTIVITY:[53,57,58]

i. Maximal electroshock seizure (MES) model -

- Presence or Absence of Tonic Hind Limb Extension
  - Tonic Hind Limb Extension is a position when tail and both hindlimbs are parallel to each other during an episode of generalized tonic clonic seizure.
- Seizure activity scoring[53] (Score 0-4) as shown below:
  - 0 – No seizure
  - 1 – Forelimb extension without hindlimb extension
  - 2 – Complete forelimb extension and partial hindlimb extension
  - 3 – Complete tonic hindlimb extension
  - 4 – Postictal depression
- Percentage protection (%) calculated as follows

$$\frac{\text{Number of animals with THLE absent}}{\text{Total number of animals}} \times 100$$

ii. Pentylenetetrazole (PTZ) model -

- Onset of seizure activity (in seconds)
- Total duration of seizure (in seconds)
- Number of seizures in one hour
- Severity of seizures[53] (Score 0-5) as shown below
  - 0.5 – Atypical behavioral changes (intense grooming, sniffing, moving arrests)

- 1 – Isolated myoclonic jerks, ear and facial twitching
- 2 – Atypical minimal seizures, convulsive wave through the body
- 3 – Fully developed minimal seizures, clonus of the head muscles and fore limbs, righting reflex present
- 4 – Major seizures (generalized without tonic phase)
- 5 – Generalized tonic-clonic seizures beginning with running

5.9. Statistical analysis

- The data were entered into the Microsoft Office Excel 2007 for Windows 7
- Data analysis was done using GraphPad Instat version 3.06, 32 bit for Windows [GraphPad Software, San Diego, California USA]
- One-way ANOVA [Parametric test] followed by Bonferroni's post hoc test was used for data with normal (Gaussian) distribution to find out the statistical significance between the study groups
- Kruskal Wallis test [Non-parametric test] followed by Dunn's post hoc test was used for data with non-normal (Non-Gaussian) distribution to find out the statistical significance between the study groups
- $P < 0.05$ was considered as statistically significant
- The results in bar diagrams are presented as Median ± SE [For scores] and Mean ± SE [For all other parameters]

RESULTS

6. Results:

6.1. Animals used in study:

In this study, 78 adult healthy Wistar albino rats of either sex were used. All the above animals were categorized into 13 groups of 6 animals in each group. The grouping of animals has been shown in Table no: 1 and 2.

6.2. Assessment of reaction time in tail clip model for evaluation of central analgesic activity: (Figure 3)

The study showed statistical significant increase in reaction time in Group II [P<0.001], Group III [P<0.01] and Group IV [P<0.001] when compared to Group I.

The increase in reaction time in Group IV was comparable to Group II, as no statistically significant difference was found between these two groups [P>0.05]. Group III was not comparable to the standard Group II, as there was statistical significant difference found among these groups [P<0.001]. The study also showed that, there was statistical significant increase in reaction time in Group IV when compared to the Group III [P<0.01].

6.3. Assessment of reaction time in hot plate model for evaluation of central analgesic activity: (Figure 4)

The study showed statistical significant increase in reaction time in Group II [P<0.001] and Group IV [P<0.01] when compared to Group I.

The increase in reaction time in Group IV was comparable to Group II, as there was no statistical significant difference between these two groups [P>0.05]. Group

III was found to be significantly different from standard Group II [P<0.001]. There was an increase in reaction time in Group III when compared to Group I, but it was not found to be statistically significant [P>0.05]. The study also showed that, there was significant rise in reaction time in Group IV when compared to the Group III [P<0.05].

6.4. Assessment of total number of writhes [in 30 minutes] in 4% Nacl induced model for evaluation of peripheral analgesic activity: (Figure 5)

The study showed statistically significant reduction in total number of writhes in Group II [P<0.01], Group IV [P<0.05] and Group V [P<0.01] when compared to Group I. There was decline in total number of writhes in Group III when compared to Group I, however it was not statistically significant [P>0.05].

The decrease in total number of writhes in Group IV was comparable to Group II and Group V, as there was no statistical significant difference was found between these groups [P>0.05].

6.5. Assessment of percent inhibition of total number of writhes [in 30 minutes] in 4% Nacl induced model for evaluation of peripheral analgesic activity: (Figure 6)

The percent inhibition of total number of writhes [in 30 minutes] in different groups includes Group I [0.00%], Group II [87.85%], Group III [68.23%], Group IV [82.87%] and Group V [85.36%].

6.6. Assessment of scores of seizures in maximal electroshock seizure model: (Figure 7)

The study showed statistically significant decrease in scores of seizures in Group VII [P<0.001] and Group IX [P<0.001] when compared to Group VI. The decrease in scores of seizures in Group IX was comparable to the Group VII, as no statistical significant difference was found among these groups [P>0.05].

There was also decrease in scores of seizures in Group VIII when compared to Group VI, but it was not found statistically significant [P>0.05].

6.7. Assessment of the percentage of protection of Tonic Hind Limb Extension [THLE] in maximal electroshock seizure model: (Figure 8)

In this study, all the animals in Group VI had tonic hind limb extension, however, none of the animals in Group VII, Group VIII and Group IX exhibited tonic hind limb extension. The percentage of protection of tonic hind limb extension in Group VII, Group VIII and Group IX was 100% in all these three groups.

6.8. Assessment of onset of seizures in pentylenetetrazole model: (Figure 9)

The study showed statistical significant increase in onset of seizure time in Group XI [P<0.001] and Group XIII [P<0.001] when compared to Group X. The increase in onset of seizure time in Group XIII was comparable to Group XI, as there was no statistical significant difference between these two groups [P>0.05].

There was a statistical significant difference between Group XI and XII [P<0.001]. There was also statistical significant difference found between Group XII and XIII

[P<0.01]. There was also increase in onset of seizure time in Group XII when compared to Group X, however it was not found statistically significant [P>0.05].

6.9. Assessment of duration of seizures in pentylenetetrazole model: (Figure 10)

The study showed statistical significant decline in duration of seizure time in Group XI [P<0.001] and Group XIII [P<0.05] when compared to Group X. There was statistical significant difference between Group XII when compared to Group XI [P<0.05].

There was also decline in duration of seizure time in Group XII when compared to Group X, however it was not found to be statistically significant [P>0.05].

The decrease in duration of seizure time in Group XIII was comparable to Group XI, as there was no statistical significant difference found between these two groups [P>0.05].

6.10. Assessment of number of seizures [in 1 hour] in pentylenetetrazole model: (Figure 11)

The study showed statistical significant reduction in number of seizures observed in 1 hour in Group XI [P<0.001] and Group XIII [P<0.05] when compared to Group X.

There was also reduction in number of seizures in 1 hour in Group XII when compared to Group X, however it was not found statistically significant [P>0.05]. No statistical significant difference was found between Group XIII when compared to Group XI [P>0.05].

6.11. Assessment of scores of seizures in pentylenetetrazole model: (Figure 12)

The study showed statistically significant decrease in scores of seizures in Group XI when compared to Group X [P<0.01].

There was also decrease in scores of seizures in Group XII [P>0.05] and Group XIII [P>0.05] when compared to Group X, however it was not found statistically significant. There was also decrease in scores of seizures in Group XIII when compared to Group XII, however it was not found statistically significant [P>0.05].

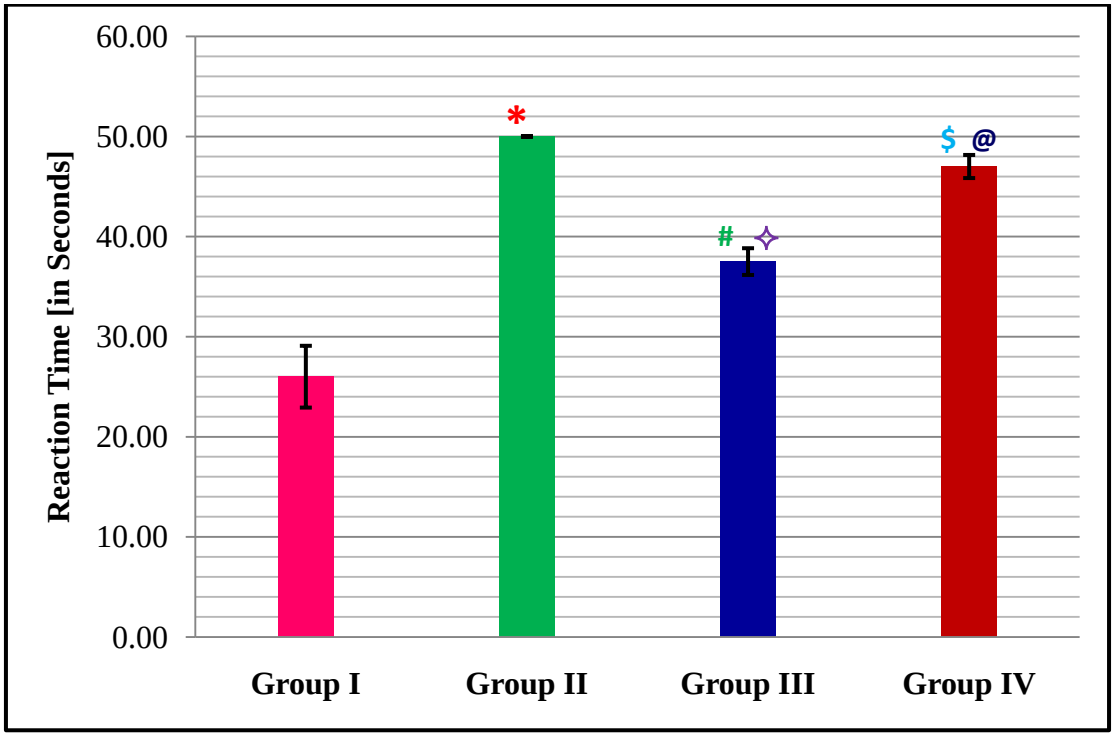


Figure 3. Bar diagram showing the reaction time [seconds] in tail clip model of central analgesic activity among different groups in Wistar Albino rats

Data are represented as **Mean ± SE**
n = 6 in each group
*P < 0.001 when compared to group I
#P < 0.01 when compared to group I
$P < 0.001 when compared to group I
✧P < 0.001 when compared to group II
@P < 0.01 when compared to group III

Group I: Sterile water equivalent volume orally [Vehicle control for tail clip method, hot plate method and writhing test]

Group II: Morphine 5 mg/Kg body weight intraperitoneally [Positive control for tail clip method, hot plate method and writhing test]

Group III: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Group IV: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Data are analysed by One-way ANOVA with Bonferroni's post-hoc test

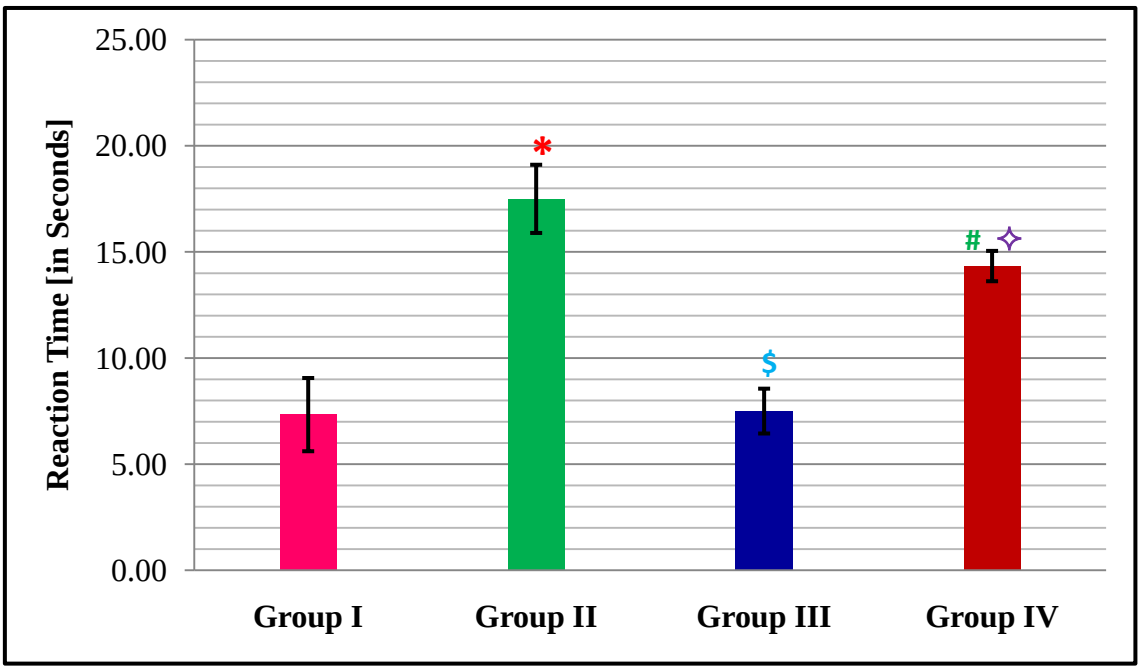


Figure 4. Bar diagram showing the reaction time [seconds] in hot plate model of central analgesic activity among different groups in Wistar Albino rats

Data are represented as **Mean ± SE**

n = 6 in each group

*P < 0.001 when compared to group I

#P < 0.01 when compared to group I

$P < 0.001 when compared to group II

✧P < 0.05 when compared to group III

Group I: Sterile water equivalent volume orally [Vehicle control for tail clip method, hot plate method and writhing test]

Group II: Morphine 5 mg/Kg body weight intraperitoneally [Positive control for tail clip method, hot plate method and writhing test]

Group III: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Group IV: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Data are analysed by One-way ANOVA with Bonferroni's post-hoc test

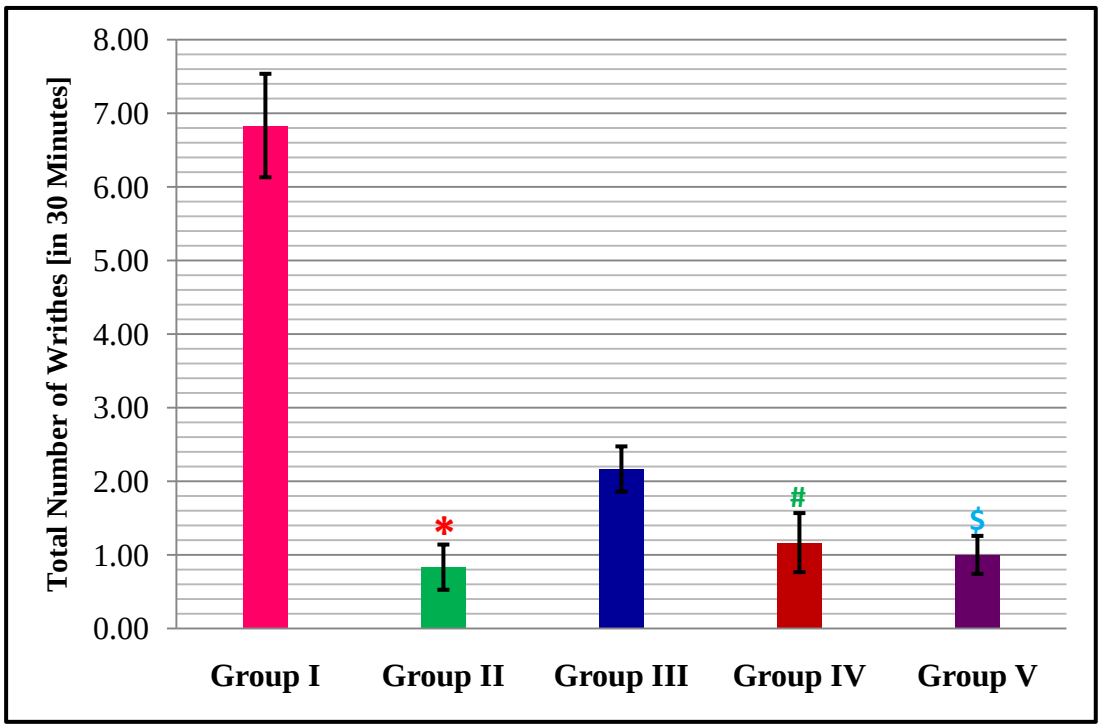


Figure 5. Bar diagram showing the total number of writhes in 30 minutes in 4% NaCl induced model of peripheral analgesic activity among different groups in Wistar Albino rats

Data are represented as **Mean ± SE**

n = 6 in each group

***P** < 0.01 when compared to group I

$^{\#}$P < 0.01 when compared to group I

$^{\$}$P < 0.01 when compared to group I

Group I: Sterile water equivalent volume orally [Vehicle control for tail clip method, hot plate method and writhing test]

Group II: Morphine 5 mg/Kg body weight intraperitoneally [Positive control for tail clip method, hot plate method and writhing test]

Group III: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Group IV: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally

Group V: Diclofenac sodium 12.5 mg/kg body weight orally [Positive control for writhing test]

Data are analysed by non-parametric Kruskal Wallis test with Dunn's post-hoc test

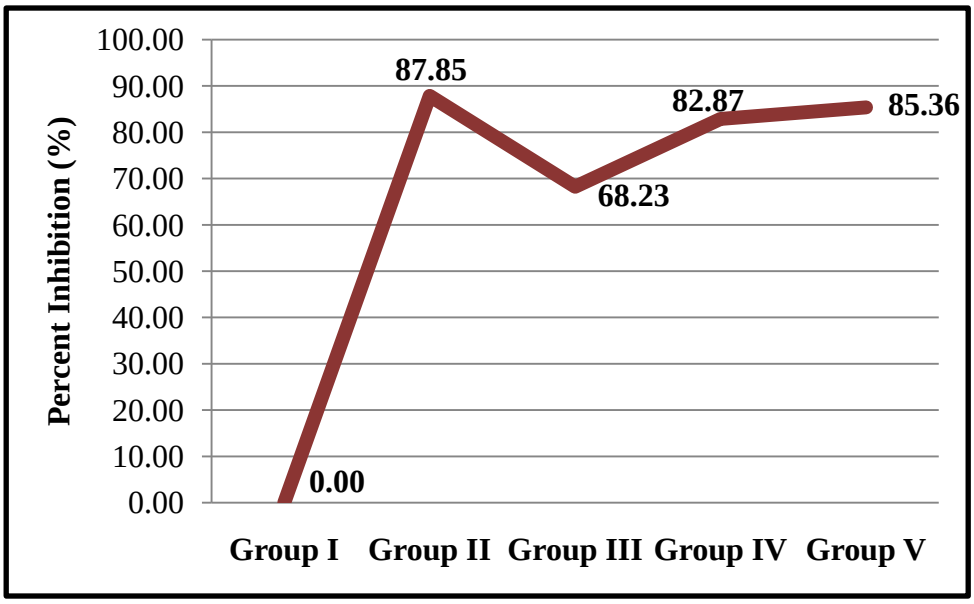


Figure 6. Line diagram showing the percent inhibition of total number of writhes in 30 minutes in 4% NaCl induced model of peripheral analgesic activity among different groups in Wistar Albino rats

n = 6 in each group

% Inhibition was calculated by $\frac{\text{Average writhes (Control)} - \text{Average writhes (Test)}}{\text{Average writhes (Control)}} \times 100$

Group I: Sterile water equivalent volume orally [Vehicle control for tail clip method, hot plate method and writhing test]

Group II: Morphine 5 mg/Kg body weight intraperitoneally [Positive control for tail clip method, hot plate method and writhing test]

Group III: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For tail clip method, hot plate method and writhing test]

Group IV: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally

Group V: Diclofenac sodium 12.5 mg/kg body weight orally [Positive control for writhing test]

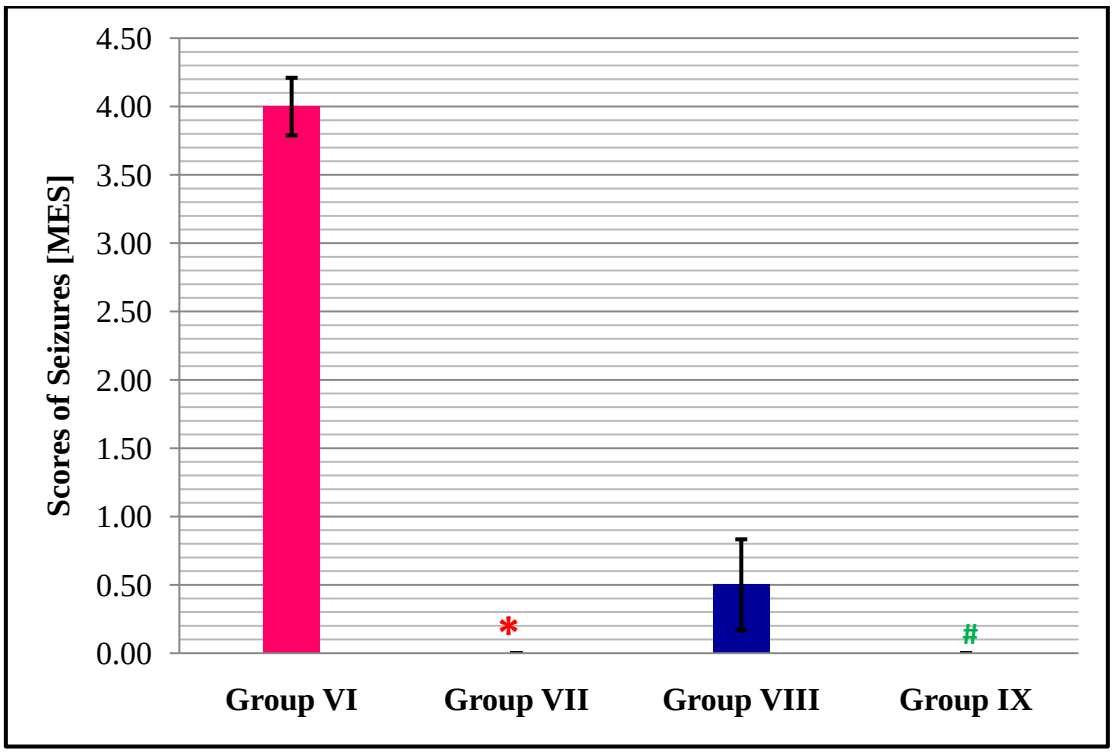


Figure 7. Bar diagram showing the scores of seizures in Maximal Electroshock Seizure [150 mA current at frequency of 60 Hz, for duration of 0.2 sec] electrical model in Wistar Albino rats

Data are represented as **Median± SE**

n = 6 in each group

*****P < 0.001 when compared to group VI

[#]P < 0.001 when compared to group VI

Group VI: Sterile water equivalent volume orally [Vehicle control for MES model]

Group VII: Phenytoin 10 mg/kg body weight intraperitoneally [Positive control for MES model]

Group VIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For MES model]

Group IX: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally [For MES model]

Score 0: No seizure
Score 1: Forelimb extension without hindlimb extension
Score 2: Complete forelimb extension and partial hindlimb extension
Score 3: Complete tonic hindlimb extension [THLE]
Score 4: Postictal depression

Data are analysed by non-parametric Kruskal Wallis test with Dunn's post-hoc test

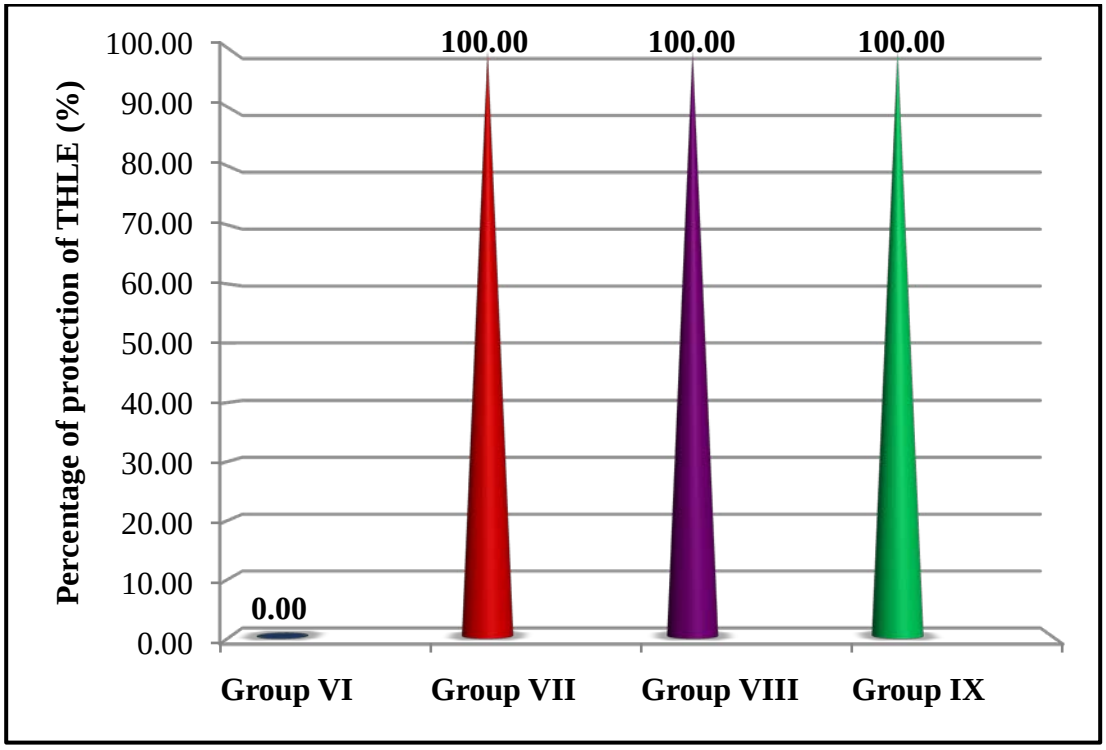


Figure 8. Bar diagram showing the percentage of protection of Tonic Hind Limb Extension [THLE] in Maximal Electroshock Seizure [150 mA current at frequency of 60 Hz, for duration of 0.2 sec] electrical model in Wistar Albino rats

Data are represented as **Percentage [%]**

n = 6 in each group

Percentage protection was calculated by $\frac{\text{Number of animals with THLE absent}}{\text{Total number of animals}} \times 100$

Group VI: Sterile water equivalent volume orally [Vehicle control for MES model]

Group VII: Phenytoin 10 mg/kg body weight intraperitoneally [Positive control for MES model]

Group VIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For MES model]

Group IX: *Costus pictus* hydroalcoholic extract 400 mg/Kg body weight orally [For MES model]

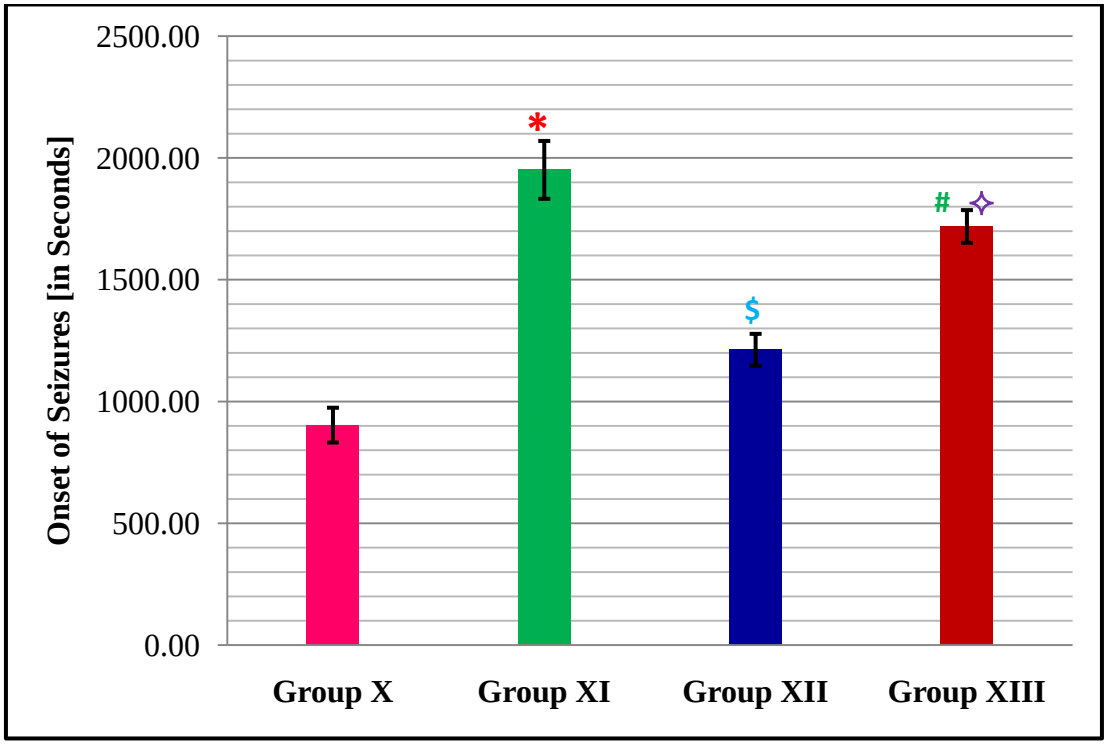


Figure 9. Bar diagram showing the onset of seizures in a chemical model in Wistar Albino rats [Pentylenetetrazole 70 mg/Kg by subcutaneous route]

Data are represented as **Mean ± SE**

n = 6 in each group

*P < 0.001 when compared to group X

#P < 0.001 when compared to group X

$P < 0.001 when compared to group XI

✧P < 0.01 when compared to group XII

Group X: Sterile water equivalent volume orally [Vehicle control for PTZ model]

Group XI: Sodium valproate 400 mg/kg body weight intraperitoneally [Positive control for PTZ model]

Group XII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Group XIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Data are analysed by One-way ANOVA with Bonferroni's post-hoc test

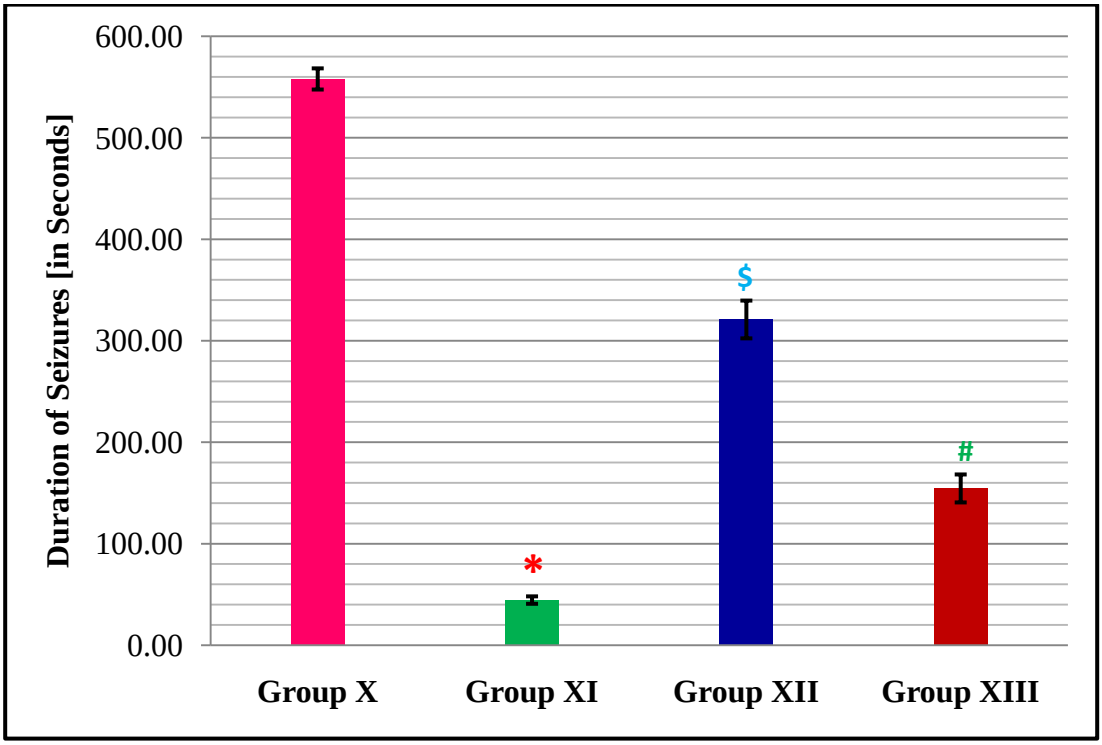


Figure 10. Bar diagram showing the duration of seizures in a chemical model in Wistar Albino rats [Pentylenetetrazole 70 mg/Kg by subcutaneous route]

Data are represented as **Mean ± SE**

n = 6 in each group

***P** < 0.001 when compared to group X

$^{\#}$P < 0.05 when compared to group X

$^{\$}$P < 0.05 when compared to group XI

Group X: Sterile water equivalent volume orally [Vehicle control for PTZ model]

Group XI: Sodium valproate 400 mg/kg body weight intraperitoneally [Positive control for PTZ model]

Group XII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Group XIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Data are analysed by non-parametric Kruskal Wallis test with Dunn's post-hoc test

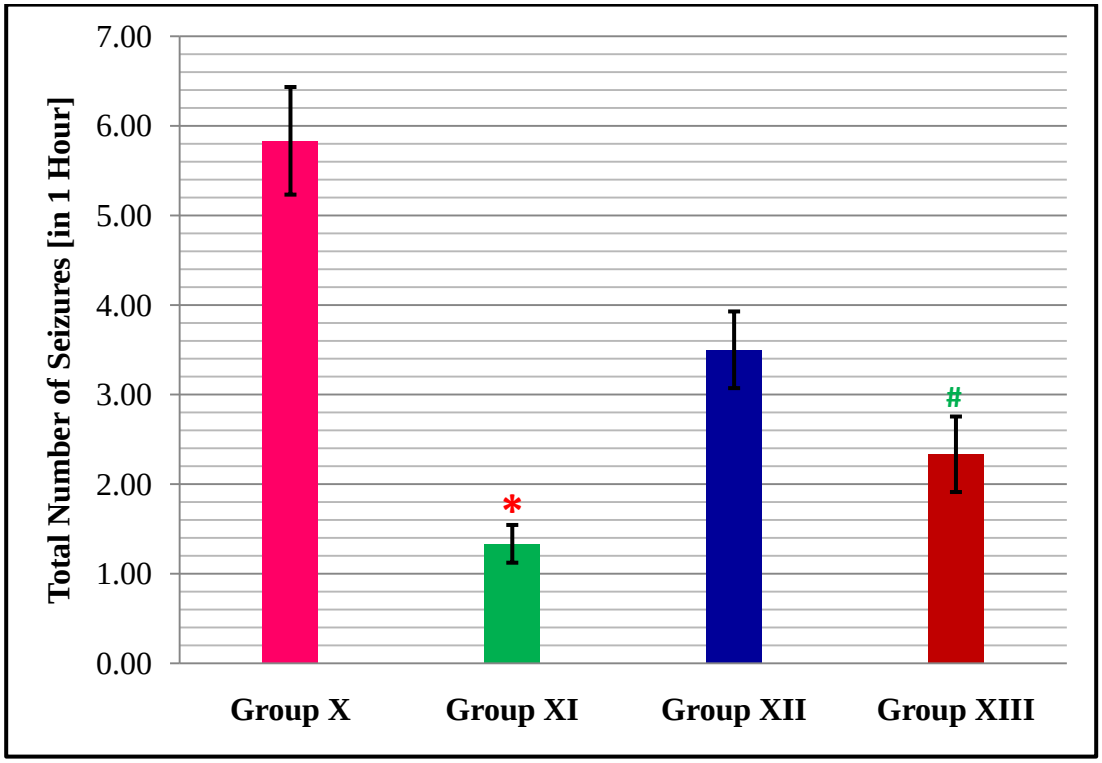


Figure 11. Bar diagram showing the total number of seizures in a chemical model in Wistar Albino rats [Pentylenetetrazole 70 mg/Kg by subcutaneous route]

Data are represented as **Mean ± SE**

n = 6 in each group

***P** < 0.001 when compared to group X

[#]P < 0.05 when compared to group X

Group X: Sterile water equivalent volume orally [Vehicle control for PTZ model]

Group XI: Sodium valproate 400 mg/kg body weight intraperitoneally [Positive control for PTZ model]

Group XII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Group XIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Data are analysed by non-parametric Kruskal Wallis test with Dunn's post-hoc test

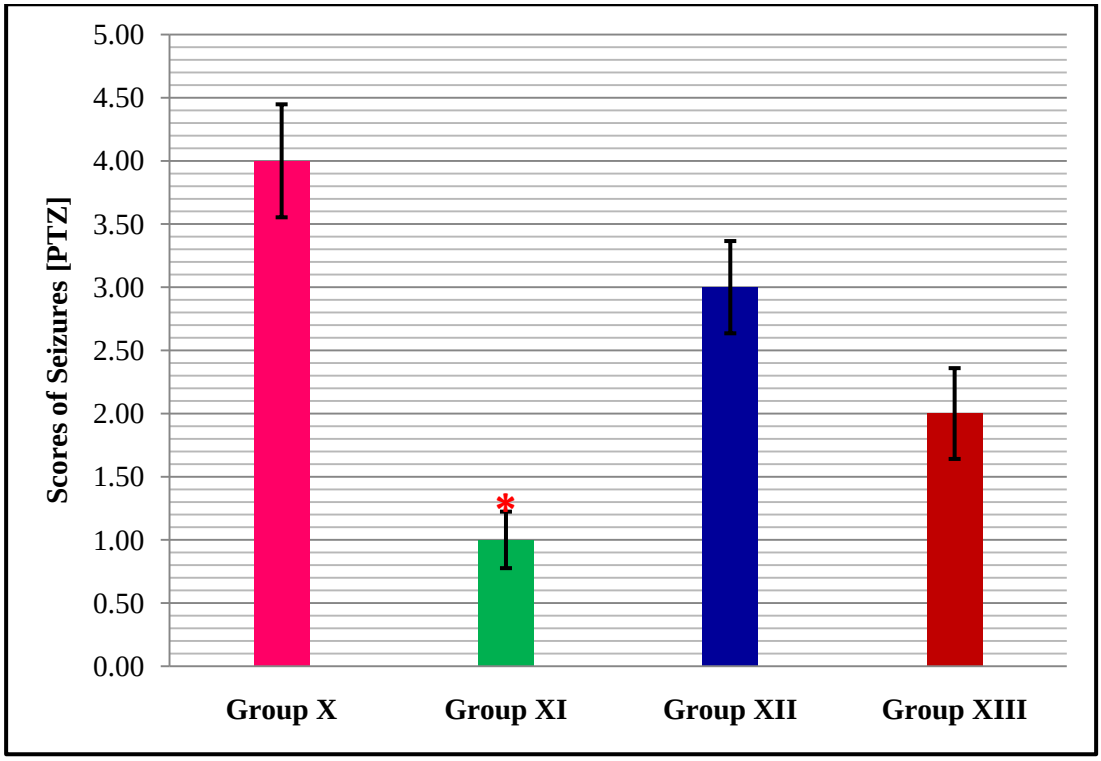


Figure 12. Bar diagram showing the scores of seizures in a chemical model in Wistar Albino rats [Pentylenetetrazole 70 mg/Kg by subcutaneous route]

Data are represented as **Mean ± SE**

n = 6 in each group

***P** < 0.01 when compared to group X

Score 0: No behavioral changes

Score 1: Isolated Myoclonic jerks, ear and facial twitching

Score 2: Atypical minimal seizures, convulsive wave through the body

Score 3: Fully developed minimal seizures, clonus of head muscles & forelimbs, righting reflex present

Score 4: Major seizures [Generalized without tonic phase]

Score 5: Generalized tonic-clonic seizures beginning with running

Group X: Sterile water equivalent volume orally [Vehicle control for PTZ model]

Group XI: Sodium valproate 400 mg/kg body weight intraperitoneally [Positive control for PTZ model]

Group XII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Group XIII: *Costus pictus* hydroalcoholic extract 200 mg/Kg body weight orally [For PTZ model]

Data are analysed by non-parametric Kruskal Wallis test with Dunn's post-hoc test

DISCUSSION

7. Discussion

The present study was aimed at evaluating the analgesic and anticonvulsant activities of hydroalcoholic extract of *Costus pictus* leaves in Wistar albino rats. Central analgesia models (Haffner's tail clip and hot plate methods) and peripheral analgesia model (writhing test) were used in evaluation of analgesic effect of the test drugs in acute pain. Anticonvulsant effects of the test drugs were evaluated using electrically (MES model) and chemically (PTZ) induced acute seizure models.

In both tail clip and hot plate methods, increase in pain threshold was seen at both the doses (200 mg/Kg BW and 400 mg/Kg BW) of CP-HALE. 400 mg/Kg BW of the drug showed significantly higher increase in pain threshold and the effects were comparable with that of the standard drug used.

Writhing test though lacks specificity, gives a good correlation with the clinical potencies of the evaluated analgesics.[51] As opioid and non-opioid analgesics, antipyretics and NSAIDs can be effectively evaluated with relatively low doses using 4% NaCl, this was used in our study.[51] The present study showed there was a significant decrease in the total number of writhes in 30 minutes with CP-HALE 400 mg/Kg BW and was comparable with both the standard drugs used in the study. Compounds with percent inhibition of more than 70% are usually taken up for further dose ranging studies while those with less than 70% are considered as minimally active.[51] The percent inhibition of CP-HALE (200 mg/Kg BW) was less than 70% [68.23%] while

with 400 mg/Kg BW of the drug it was greater than 70% [82.87%]. This result was comparable with both the standard drugs used (Morphine [87.85%] and Diclofenac sodium [85.36%]). This indicates that 400 mg/Kg BW could be a suitable dose taken for further studies for evaluating analgesic activites.

The results obtained in our study are comparable with a study by Moron et al.[3] in which the analgesic activity of decoction of fresh leaves and stems were tested in mice models (tail flick method and acetic acid induced writhing test) and their results revealed a significant dose dependent pain inhibition.

The evaluation of CP-HALE for its anticonvulsant activity using MES model revealed that at both the doses (200 mg/Kg BW and 400 mg/Kg BW) there was 100 percent protection against tonic hind limb extension (THLE). A significant decline in the seizure scores was observed with 400 mg/Kg BW and this was comparable with the standard drug used. The decline in seizure scores was not significant at 200 mg/Kg BW administered dose.

In pentylenetetrazole (PTZ) model, CP-HALE at both the doses increased the time for onset of seizures and decreased the duration of seizures. These effects observed were higher and significant at 400 mg/Kg BW dose which was comparable to the standard drug used. There was also a significant reduction in the total number of seizures (in one hour) with CP-HALE 400 mg/Kg BW in the PTZ model. There was no significant decline in the seizure

scores of PTZ model with CP-HALE at both the doses (200 mg/Kg BW and 400 mg/Kg BW).

Free radicals are being implicated in the development and progression of various physiological and pathological states including pain and epilepsy.[18,19] Phytomedicines which are rich sources of antioxidants could have a significant therapeutic potential in their management. Flavanoids and phenolic compounds are the important antioxidants derived from plant products.[45] Particularly, flavanoids are considered to be ideal free radical scavengers and are even more efficient than vitamins C and E.[18]

Various parts of *Costus pictus* have been proved to have antioxidant property by several previous studies.[5,8,9] Phytochemical analyses in earlier sstudies with *Costus pictus* have revealed the presence of flavanoids.[2,5,39] Solvent extraction is the most commonly used procedure to obtain active principles from plant sources, particularly antioxidants.[67] In a previous study by Rege et al.[9] it was shown that dry hydroalcoholic extract of *Costus pictus* leaves had highest antioxidant activity when compared with other leaf extracts. Since antioxidant property is being hypothesized for its analgesic and anticonvulsant potential, hydroalcoholic extract prepared from dried leaves was chosen for this study.

GABA is a major inhibitory neurotransmitter and an important target of several conventionally used antiepileptic drugs.[45] Potentiation of GABA receptor is protective against MES and PTZ induced seizures. [45,68,69,70] PTZ

induced seizures mimics a state of increased oxidative stress in the brain.[45] Flavanoids have been found to modulate $GABA_A$-Cl channel complex and also increase the seizure threshold.[45,68,69] Hence, flavanoids by its potent antioxidant and anticonvulsant activity might be responsible for the significant effects as seen in our study.

All precautions to minimize bias due to gender or diurnal variations were taken. Male and female rats were equally distributed among the groups to minimize gender variations on the results. Pregnancy was excluded in female rats before inclusion into the study. All experiments were conducted during the same hours on all days to minimize diurnal variations.

MES and subcutaneous PTZ (scPTZ) seizure models are the commonly used models to evaluate the effects of a drug on acute seizures.[55] MES model is representative of grandmal epilepsy and scPTZ model represents generalized absence seizures in humans.[55] In this study, animal models and drugs (phenytoin for MES model and sodium valproate for PTZ model) as prescribed by standard protocols were used.[51] In particular, since PTZ is a GABA antagonist, PTZ model is sensitive to GABA mimetic drugs.[45] Since the proposed activity of our study extract may be due to the presence of flavanoids which modulates GABA, the PTZ model used in our study is very relevant.

Oxidative stress had been the basis for hypothesis for the analgesic and anticonvulsant activity of the *Costus pictus* hydroalcoholic extract. But this

study did not include any markers for evaluating the oxidative stress levels or restoration of antioxidant levels.

MES and PTZ models are acute seizure models.[55] Since epilepsy is a chronic condition requiring daily drug administration,[14] chronic drug administration could yield different results. Hence further studies with chronic models like kindling models are needed for more predictive data.

Our present study has thus confirmed that *Costus pictus* hydroalcoholic extract at 400 mg/Kg BW had better analgesic and anticonvulsant activity when compared to the 200 mg/Kg BW dose. Further animal studies using different models and larger clinical trials are needed to confirm these results.

CONCLUSION

8. Conclusion

The hydroalcoholic extract of *Costus pictus* leaves possess significant central and peripheral analgesic activity in Wistar albino rats at the dose of 400 mg/Kg BW.

Significant anticonvulsant activity on electrically induced and pentylenetetrazole induced seizures in Wistar albino rats is seen with hydroalcoholic extract of *Costus pictus* leaves at the dose of 400 mg/Kg BW.

SUMMARY

9. Summary

Plants are widely researched in search of new drugs since ancient times and there is a re-emerging interest on phytomedicines.[1] Limitations seen with the currently available drugs have always brought forth the need for a well tolerated analgesic and antiepileptic drug. Oxidative stress is an important underlying pathophysiologic process in pain and epilepsy.[18,19] *Costus pictus* widely grown as an ornamental plant is very popular for its various medicinal properties. *Costus pictus* has proven evidence in literature to have antioxidant property.[5,8,9] A study had particularly shown that the hydroalcoholic extract of *Costus pictus* obtained from dried leaves has a very high antioxidant potential[9] and this was the basis for our hypothesis. Our study was thus aimed to evaluate the analgesic and anticounvulsant properties of *Costus pictus* using appropriate models in Wistar albino rats.

A total of 78 Wistar albino rats of either sex were used in this study. The central analgesic activity of the hydroalcoholic extract of *Costus pictus* leaves (CP-HALE at doses of 200 mg/Kg BW and 400 mg/Kg BW) was evaluated using the tail clip and hot plate methods. CP-HALE was found to increase the pain threshold at both the doses. This effect was highly significant with 400 mg/Kg BW. The peripheral analgesic property was assessed using 4% NaCl induced writhing test, which revealed that CP-HALE (400 mg/Kg BW) showed highly significant reduction in number of writhes observed in 30 minutes and the percent inhibition was greater than 70%. This showed that CP-HALE 400 mg/Kg BW had good peripheral

analgesic activity which was comparable to the standard drugs used in the study.

Anticonvulsant properties were tested using MES and PTZ models which revealed better activity with CP-HALE 400 mg/Kg BW. Both the doses (200 mg/kg BW and 400 mg/Kg BW) showed 100% protection against tonic hind limb extension induced by MES model. In PTZ model, treatment with CP-HALE prolonged the time to onset of seizures, significantly decreased the duration of seizures and the total number of seizures in one hour. These effects were highly significant with CP-HALE 400 mg/Kg BW. This has brought forth the fact that CP-HALE 400 mg/Kg BW has good anticonvulsant activity and is comparable with the standard drugs used.

Flavanoids with a very high antioxidant potential have been found in various extracts of *Costus pictus* by several earlier studies.[2,5,39] Also, flavanoids have been found to modulate $GABA_A$-Cl channels which might be responsible for its anticonvulsant potential.[45,68,69] Hence the analgesic and anticounvulsant effects seen in our study could be attributed to the presence of flavanoids in the hydroalcoholic extract of *Costus pictus* leaves. From the results of our study it can be concluded that hydroalcoholic extract of *Costus pictus* leaves (CP-HALE 400 mg/Kg BW) possess significant analgesic and anticonvulsant activity. Hydroalcoholic extract of *Costus pictus* leaves (400 mg/Kg BW) can be taken up for further larger animal and human studies for more predictive results.

REFERENCES

10. References

1. Rates SMK. Plants as source of drugs. Toxicon 2001;39:603-13.
2. Thomas S, Devi BS. Phytochemical and *in-vitro* anthelmintic studies of hydro-alcoholic extract of *Costus pictus* D. Don. Int J Pharm Pharm Sci 2013;5:639-41.
3. Moron FR, Amador MCV, Morejon ZR, Barreiro ML, Garcia IHA, Fiallo VF et al. Phytochemical sreening, analgesic and antiinflammatory properties of *Costus pictus* D. Don decoction. Rev Cubana Med Plant 2008;13:1-8.
4. Jayasri MA, Gunasekaran S, Radha A, Mathew TL. Anti-diabetic effect of *Costus pictus* leaves in normal and streptozotocin-induced diabetic rats. Int J Diab Metab 2008;16:117-22.
5. Remya R, Daniel M. Phytochemical and pharmacognostic investigation of antidiabetic *Costus pictus* D. Don. Int J Pharm Biomed Res 2012;3:30-9.
6. Majumdar M, Parihar PS. Antibacterial, antioxidant and antiglycation potential of *Costus pictus* from southern region, India. Asian J Plant Sci Res 2012;2:95-101.
7. Sulakshana G, Rani AS, Saidulu B. Evaluation of antibacterial activity in three species of *Costus*. Int J Curr Microbiol App Sci 2013;2:26-30.
8. Rajasekaran M, Kalaichelvan S. Antioxidative role of *Costus pictus* D. Don in hyperglycemic rats administered with alloxan. J Pharm Sci Res 2014;6:24-8.
9. Rege AA, Ambaye RY, Chowdhary AS. Effect of *Costus pictus* D. Don on pepsin enzyme. Int J Pharm Pharm Sci 2014;6:178-80.
10. Choudhury JD, Maurya AK, Tripathi S, Ranga R, Kahrana M, Samyal ML. Estimation of the anti-proliferative potential of *Costus pictus* on MOLT-4 human cancer cell line. Int J Biopharm Toxicol Res 2012;2:244-50.
11. Yaksh TL, Wallace MS. Opioids, Analgesia and Pain management. In: Brunton LL, Chabner BA, Knollman BC editors. Goodman and Gilman's

The Pharmacological Basis of Therapeutics. 12th ed. New York: McGraw-Hill, 2011. p. 481-526.

12. Rathmell JP, Fields HL. Pain: Pathophysiology and Management. In: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J editors. Harrison's Principles of Internal Medicine Vol 2. 18th ed. United States of America: MacGraw- Hill; 2012. p. 93-101.

13. Baumann TJ, Herndon CM, Strickland JM. Pain management. In: Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM editors. Pharmacotherapy A pathophysiologic approach. 9th ed. United States of America: McGraw-Hill; 2014. p. 925-42.

14. Lowenstein DH. Seizures and Epilepsy. In: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J editors. Harrison's Principles of Internal Medicine Vol 2. 18th ed. United States of America: MacGraw-Hill; 2012. p. 3251-70.

15. McNamara JO. Pharmacotherapy of the epilepsies. In: Brunton LL, Chabner BA, Knollman BC editors. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 12th ed. New York: McGraw-Hill, 2011. p. 583-608.

16. Rogers SJ, Cavazos JE. Epilepsy. In: Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM editors. Pharmacotherapy A pathophysiologic approach. 9th ed. United States of America: McGraw-Hill; 2014. p. 855-82.

17. Folbergrova J. Oxidative stress in immature brain following experimentally-induced seizures. Physiol Res 2013;62:S39-48.

18. Aguiar CCT, Almeida AB, Araujo PVP, Abreu RNDCD, Chaves EMC, Vale OCD, et al. Oxidative stress and epilepsy: Literature review. Oxid Med Cell Longevity. [Online]. 2015 September 4 [cited 2015 September 4]; Available from: URL: http://www.hindawi.com/journals/omcl/2012/795259/

19. Taha R, Blaise GA. Update on the pathogenesis of complex regional pain syndrome: role of oxidative stress. [Online]. 2015 July 23 [cited 2015 July

23]; Available from: URL: http://www.ncbi.nlm.nih.gov/pubmed/22798149

20. Inamr A, Sogut E, Ayan M, Inamr S. Evauation of pain intensity and oxidative stress levels in patients with inflammatory and non-inflammatory back pain. Eur J Gen Med 2013;10:185-90.

21. Rodriguez NC, Gertrudis BH, Espinosa LR, Correa HM, Bandala C, Aparicio LC et al. Role of oxidative stress in refractory epilpepsy: Evidence in patients and experimental models. Int J Mol Sci 2013;14:1455-76.

22. Patil CD, Ahire YS, Pathade PA, Pathade VV, Mali PR. Free radicals, epilepsy and anti-Oxidant: An overview. Int Res J Pharm 2011;2:64-71.

23. Sarker SD. Pharmacognosy in modern pharmacy curricula. Pharmacogn Mag 2012;8:91-2.

24. Specht CD, Kress WJ, Stevenson DW, DeSalle R. A molecular phylogeny of *Costaceae (Zingiberales)*. Mol Phylogen Evol 2001;21:333-45.

25. Benny M. Insulin Plant in Gardens. Nat Prod Radiance 2004;3:349-50.

26. Jose B, Reddy LJ. Analysis of the essential oils of the stems, leaves and rhizomes of the medicinal plant *Costus Pictus* from southern India. Int J Pharm Pharm Sci 2010;2:100-1.

27. Pareek A, Suthar M, Godavarthi A, Goyal M, Bansal V. Negative regulation of glucose uptake by Costus pictus in L6 myotube cell line. J Pharm Negat Results 2010;1:24-6.

28. Punyarani K, Sharma JG. Micropropagation and microrhizome induction in *Costus pictus* D. Don using *in vitro* and *ex vitro* nodal segments as explant. Not Sci Biol 2012;4:72-8.

29. Shiny CT, Saxena A, Gupta SP. Phytochemical and hypoglycaemic activity investigation of *Costus pictus* plants from Kerala and Tamilnadu. Int J Pharm Sci Invent 2013;2:11-8.

30. Sidhu AK, Wani SJ, Tamboli PS, Patil SN. *In vitro* evaluation of anti-diabetic activity of leaf and callus extracts of *Costus pictus*. Int J Sci Res 2014;3:1622-5.

31. Aruna A, Nandhini A, Karthikeyan V, Bose P, Vijayalakshmi K. Comparative anti-diabetic effect of methanolic extract of insulin plant (*Costus pictus)* leaves and its silver nanoparticle. Indo Am J Pharm Res 2014;4:3217-30.

32. *Costus pictus* D.Don, Edwards's Bot. Reg. 19: t. 1594 (1833). [Online]. 2015 September 3 [cited 2015 September 3]; Available from: URL: http://apps.kew.org/wcsp/namedetail.do?name_id=234724

33. Reddy LJ, Jose B. Evaluation of antibacterial activity of the leaf essential oil of *Costus pictus* D. Don from south India. Int J Current Pharm Res 2010;2:68-70.

34. Hegde PK, Rao HA, Rao PN. A review on insulin Plant (*Costus igneus* Nak). Pharmacogn Rev 2014;8:67-72.

35. Kumawat U, Shimpi S. Pharmacognostic studies of the *Costus Pictus D. Don.* J Herbal Med Toxicol 2009;3:127-30.

36. Sulakshana G, Rani AS. HPLC analysis of diosgenin in three species of *Costus*. Int J Pharm Sci Res 2014;5:747-9.

37. Specht CD, Stevenson DW. A new phylogeny-based generic classification of *Costaceae (Zingiberales).* Taxon 2006;55:153-63.

38. George A, Thankamma A, Devi VKR, Fernandez A. Phytochemical investigation of insulin plant (*Costus pictus*). Asian J Chem 2007;19:3427-30.

39. Shiny CT, Saxena A, Gupta SP. Phytochemical investigation of the insulin plant "*Costus pictus*" D. Don. Int J Pharm Biomed Res 2013;4:97-104.

40. Suganya S, Narmadha R, Gopalakrishnan VK, Devaki K. Hypoglycemic Effect of *Costus pictus* D. Don on Alloxan Induced Type 2 Diabetes Mellitus in Albino Rats. Asian Pac J Trop Dis 2012;2:117-23.

41. Sethumathi PP, Nandhakumar J, Sengottuvelu S, Duraisamy R, Karthikeyan D, Ravikumar VR et al. Antidiabetic and antioxidant activity of methanolic leaf extracts of *Costus pictus* D. Don in alloxan induced diabetic rats. Pharmacolonline 2009;1:1200-13.

42. Sathuvan M, Vignesh A, Thangam R, Palani P, Rengasamy R, Murugesan K. *In-vitro* antioxidant and anticancer potential of bark of *Costus pictus* D. Don. Asian Pacific J Tropical Biomed 2012;2:S741-9.

43. Manjula AC, Shubha. Evaluation of heat stable proteins, total soluble proteins and antibacterial properties of *Costus pictus* D. Don. The Bioscan 2010;5:169-70.

44. Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol 2001;54:176-86.

45. Diniz TC, Silva JC, Lima-Saraiva SRG, Ribeiro FPRA, Pacheco AGM, Freitas RM et al. The role of flavanoids on oxidative stress in epilepsy. [Online]. 2015 Aug 18 [cited 2015 Aug 18]; Available from: URL: http://www.hindawi.com/journals/omcl/2015/171756/abs/

46. Waldbaum S, Patel M. Mitochondrial oxidative stress in temporal lobe epilepsy. Epilepsy Res 2010;88:23–45.

47. Waldbaum S, Liang L, Patel M. Persistent impairment of mitochondrial and tissue redox status during lithium-pilocarpine-induced epileptogenesis. J Neurochem 2010;115:1172-82.

48. Milind P, Monu Y. Laboratory models for screening analgesics. Int res J Pharm 2013;4:15-9.

49. Bars DL, Gozariu M, Cadden SW. Animal Models of Nociception. Pharmacol Rev 2001;53:597-652.

50. Olessen AE, Anderson T, Staahl C, Drewes AM. Human experimental pain models for assessing the therapeutic efficacy of analgesic drugs. Pharmacol Rev 2012;64:722-79.

51. Vogel HG. Analgesic, Anti-Inflammatory, and Anti-Pyretic Activity. In: Drug Discovery and Evaluation: Pharmacological assays Vol 2. 3rd ed. New York: Springer; 2008. p. 983-1115.

52. Gupta SK. Analgesic agents. In: Drug Screening Methods (Preclinical Evaluation of New Drugs). 2nd ed. New Delhi: Jaypee Brothers Medical Publishers (P) Ltd; 2009. p. 461-79.

53. Medhi B, Prakash A. Animal Experiment on Central Nervous System. In: Practical Manual of Experiment and Clinical Pharmacology. 1st ed. New Delhi: Jaypee Brothers Medical Publishers (P) Ltd; 2010. p. 183-206.

54. Raina GS. Experimental (*In-Vivo*) models for assessing analgesic activity: A recent approach. Int J Recent Adv Pharm Res 2013;3:21-7.

55. White HS. Preclinical development of antiepileptic drugs: past, present, and future directions. Epilepsia 2003;44:2-8.

56. Sarkisian MR. Overview of the current animal models for human seizure and epileptic disorders. Epilepsy Behav 2001;2:201-16.

57. Vogel HG. Psychotropic and Neurotropic Activity. In: Drug Discovery and Evaluation: Pharmacological assays Vol 1. 3rd ed. New York: Springer; 2008. p. 565-874.

58. Gupta SK. Antiepileptics. In: Drug Screening Methods (Preclinical Evaluation of New Drugs). 2nd ed. New Delhi: Jaypee Brothers Medical Publishers (P) Ltd; 2009. p. 400-22.

59. Loscher W. Animal models of epilepsy for the development of antiepileptogenic and disease-modifying drugs. A comparison of the pharmacology of kindling and post-status epilepticus models of temporal lobe epilepsy. Epilepsy Res 2002;50:105-23.

60. Loscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. Seizure 2011;20:359-68.

61. Loscher W. Current status and future directions in the pharmacotherapy of epilepsy. Trends Pharmacol Sci 2002;23:113-8.

62. Standard Operating Procedures (SOP) for IAEC. [Online]. 2015 Aug 20 [cited 2015 Aug 20]; Available from: URL: https://www.aaalac.org/resources/SOP_CPCSEA.pdf

63. Mwambete KD. The *in-vitro* antimicrobial activity of fruit and leaf crude extracts of *Momordica charantia*: A Tanzania medicinal plant. African Health Sci 2009;9:34-9.

64. Mulla WA, Salunkhe VR, Bhise SB. Hepatoprotective activity of hydroalcoholic extract of leaves of *Alocasia indica* (Linn.). Ind J Exp Biol 2009;47:816-21.

65. Minz S, Samuel CO, Tripathi SC. The effect of plant extracts on the growth of wilt causing fungi *Fusarium oxysporum*. Int J Pharm Bio Sci 2012;4:13-6.

66. Tanwer BS, Choudhary S, Vijayvergia R. *In vitro* anti-inflammatory, analgesic and acute toxicity studies of ethanol extracts of *Andrographis paniculata Nees* and *Spilanthus acemella Murr*. Der Pharmacia Sinica 2012;3:590-3.

67. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 2009;14:2167-80.

68. Campbell EL, Chebib M, Johnston GAR. The dietary flavonoids apigenin and epigallocatechin gallate enhance the positive modulation by diazepam of the activation by GABA of recombinant $GABA_A$ receptors. Biochem Pharmacol 2004;68:1631-8.

69. Singh P, Singh D, Goel RK. Phytoflavonoids: Antiepileptics for the future. Int J Pharm Pharm Sci 2014;8:51-66.

70. Dhivya D, Babu MN. Anticonvulsant activity of the crude flavonoid fraction of *Pithecellobium dulce* leaf. Asian J Phytomed Clin Res 2013;1:160-6.

ANNEXURES

Sree Mookambika Institute of Medical Sciences
Kulasekharam (K.K District, TN) 629161

Phone No: 04651-280866, Fax No. 04651-280740

Institutional Animal Ethics Committee

Registered under CPCSEA with Reg No. 1144/ac/07/CPCSEA

Ref. No. SMIMS/IAEC/2014/C/01 **Date: 29th September 2014**

Certificate

This is to certify that the Research Protocol Ref. No. **SMIMS/IAEC/2014/C/01,** entitled "Evaluation of Analgesic and Anticonvulsant Activity of Hydro-alcoholic Extract of *Costus pictus* Leaves in Wistar Albino Rats" submitted by Dr. Anandhalakshmi A, Postgraduate of Department of Pharmacology, SMIMS has been approved by the Institutional Animal Ethics Committee at its meeting held on 29th of September 2014.

[This Institutional Animal Ethics Committee is organized and operates according to the requirements of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines, Ministry of Environment and Forests, Government of India.]

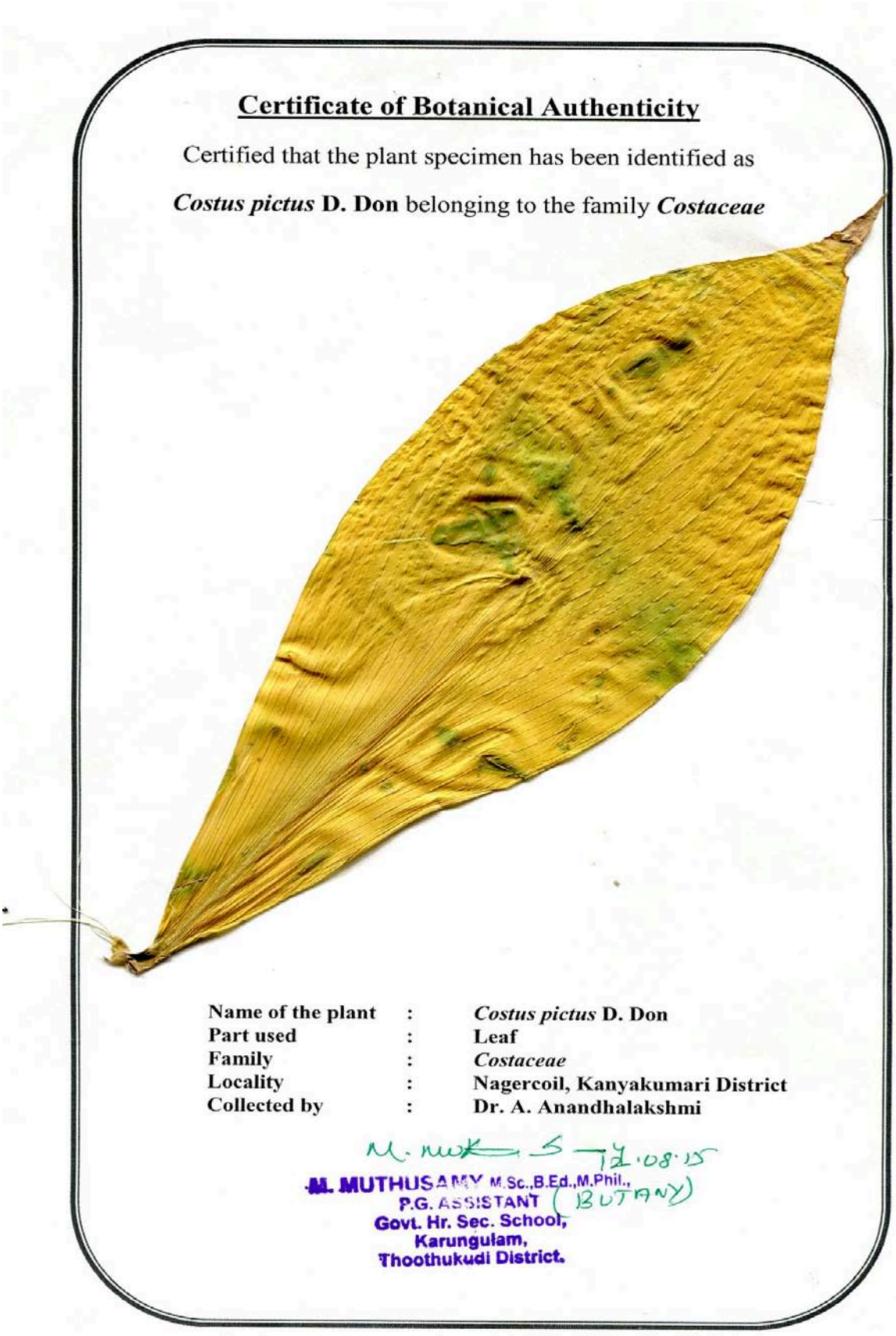
SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES • KULASEKHARAM • Date: 29/09/14

Dr. Vijay Pal Bhalla
Member Secretary
Institutional Animal Ethics Committee
SMIMS, Kulasekharam [K.K District]

Dr. J. C Stephenson
Member and CPCSEA Main Nominee
Institutional Animal Ethics Committee
SMIMS, Kulasekharam [K.K District]

Image 1: *Costus pictus* plant

Image 2: Authentication of *Costus pictus* leaf



Certificate of Botanical Authenticity

Certified that the plant specimen has been identified as

Costus pictus* D. Don** belonging to the family ***Costaceae

Name of the plant	:	***Costus pictus* D. Don**
Part used	:	**Leaf**
Family	:	***Costaceae***
Locality	:	**Nagercoil, Kanyakumari District**
Collected by	:	**Dr. A. Anandhalakshmi**

12.08.15

M. MUTHUSAMY M.Sc.,B.Ed.,M.Phil.,
P.G. ASSISTANT (BOTANY)
Govt. Hr. Sec. School,
Karungulam,
Thoothukudi District.

Image 3: Hydroalcoholic extract of *Costus pictus* leaves

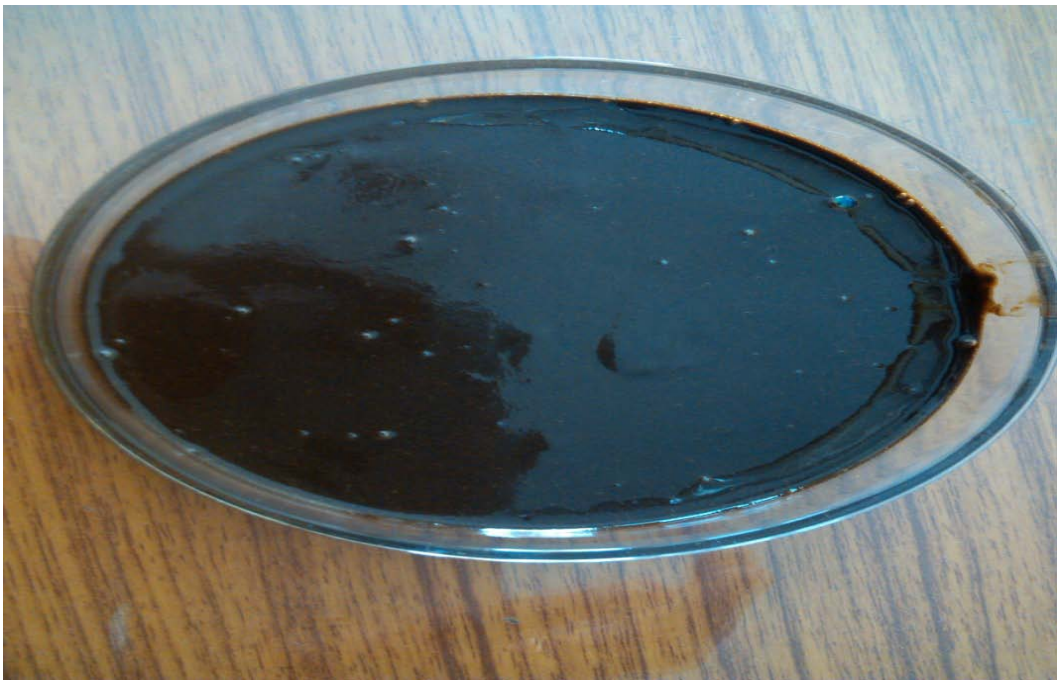


Image 4: Drugs used for evaluating analgesic activity

Image 5: Electroconvulsiometer

Image 6: Drugs and chemicals used for evaluating anticonvulsant activity

List of abbreviations	
µA	Micro Amperes
µg	Micro Grams
µL	Micro Liters
^{o}C	Degree Celsius
A/G	Albumin Globulin Ratio
AEDs	Anti Epileptic Drugs
ALP	Alkaline Phosphatase
ALT	Alanine amino transferase
ANOVA	Analysis of Variance
AST	Aspartate amino transferase
BW	Body Weight
Ca	Calcium
CAT	Catalase
CD_{97}	Convulsive Dose in 97% of the animals
Cl^-	Chloride
cm	Centimeters
CPE	*Costus pictus* ethanol extract
CP-HALE	*Costus pictus* Hydroalcoholic Leaf Extract
CPRS	Complex Pain Regional Syndrome
Cr	Chromium
Cu	Copper
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl hydrazyl
Fe	Iron
g	Grams
GABA	Gamma Amino Butyric Acid
GC-MS	Gas Chromatography Mass Spectrometry
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
GTCS	Generalized Tonic Clonic Seizures
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
HSP	Heat Stable Proteins
Hz	Hertz
i.p.	Intraperitoneally

K	Potassium
Kg	Kilo Grams
L	Liters
LDH	Lactate dehydrogenase
LDL	Low Density Lipoprotein
mA	Milliamperes
MDA	Malondialdehyde
MES	Maximal Electroshock induced Seizures
mg	Milligrams
MIC	Minimum Inhibitory Concentration
mL	Milliliters
mm	Millimeters
Mn	Manganese
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mV	Millivolts
NaCl	Sodium Chloride
Nrf2	Nuclear factor erythroid 2-related factor
NSAIDs	Non steroidal Anti-Inflammatory Agents
PTZ	Pentylenetetrazole
ROS	Reactive Oxygen Species
s.c	Subcutaneous
scPTZ	Subcutaneous Pentylenetetrazole
SE	Standard Error
sec	Seconds
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SOD	Superoxide Dismutase
TBARS	Thiobarbituric acid reactive substances
THLE	Tonic Hind Limb Extension
TLC	Thin Layer Chromatography
TSP	Total Soluble Proteins
V	Volts
v/v	Volume by Volume
VLDL	Very Low Density Lipoprotein
Zn	Zinc